Modulation of Immune Cell Responses by Small Cell Lung Cancer Cells

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I confirm that the work submitted in this thesis is my own.

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Abstract

Small Cell Lung Cancer (SCLC) accounts for 15-20% of all lung cancers and kills at least one person every 2 hours in the UK. There is no effective treatment and overall 2-year survival is less than 5%. Patients with SCLC have poorly understood local and systemic immune defects. Previous studies have shown several important defects in cell-mediated immune responses in patients with SCLC. A better understanding of interactions between SCLC tumour cells and immune cells may lead to the development of novel therapeutic approaches. There is increasing recognition that immunological biomarkers may add to traditional histological analyses and can be exploited in the management of multiple epithelial malignancies. There are currently no such markers used in the management of SCLC.

In my PhD project, I have shown that cell lines from different SCLC patients have differential immunosuppressive capabilities. These properties are mediated by the secretion of differing levels of soluble molecules that can suppress the mixed leukocyte reaction (MLR) and $CD4^+$ T cell proliferation, induce IL-10 secretion and differentiation of functional $CD4^+CD25^+CD127^+FoxP3^+Helios^-$ regulatory T cells (Tregs) from naïve $CD4^+$ T cells. IL-15 is secreted by SCLC cells in culture in proportion to their immunosuppressive capability. Its in vivo relevance is supported by its presence in tumour biopsy samples. The suppressive effect on $CD4^+$ T cell proliferation and the induction of Treg cell population was not affected by blocking IL-10 or TGF-β signalling but was partially reversed by blocking IL-15 activity. Therefore, IL-15 is one, though not the only, soluble molecule produced

by SCLC cells to mediate immune suppression by inducing increased population of Treg cells. This may represent a mechanism by which SCLC cells can suppress the immune response.

In addition, SCLC cells supressed TNF-α release from monocytes in response to LPS stimulation, down-regulated expression of CD16 and CD86 and up-regulated expression of CD163 and CD206 on monocyte-derived macrophages (MDMs) upon activation. This M2-like phenotype poralization was associated with decreased TNF-α and IL-6 production and increased IL-10 secretion. These effects were abrogated by blocking the signalling of bombesin-like peptides (BLPs) that are neuropeptides produced by SCLC cells using a GRP receptor (GRP-R) antagonist. Therefore, the polarization of macrophages to an M2-like phenotype by SCLC cell-derived BLPs may represent another mechanism by which SCLC tumours suppress the immune response.

Finally, SCLC tumour biopsies were shown to be infiltrated with various mononuclear immune cells and Treg cells. CD45 and FoxP3 were used as pan-inflammatory cell and Treg cell markers respectively. An elevated $CD45^+$ infiltrate was predictive of prolonged survival in SCLC independent of age, sex, stage or treatment strategy. An elevated $FoxP3^+/CD45^+$ ratio was predictive of a significantly worse prognosis.

This study identifies potential mechanisms by which SCLC tumour cells may downregulate local and systemic immune response, and also identifies an independent prognostic marker to predict patient survival in SCLC. Further, IL-15 and BLPs are potential novel therapeutic targets in SCLC.

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Abbreviations

AC	Adenylyl Cyclase
AM	Alveolar Macrophages
ANOVA	Analysis of variance
Apc	Allophycocyanin (Flow Cytometry Fluorochromes)
APC	Antigen Presenting Cells
ARG	Arginase
bFGF	basic Fibroblast Growth Factor
BB3	Bombesin receptor subtype 3
BSA	Bovine Serum Albumin
BLP	Bombesin-like Peptide
CAE	Cyclophosphamide, Doxorubicin and Etoposide
CAF	Cancer Associated Fibroblast
cAMP	cyclic Adenosine Monophosphate
CAV	Cyclophosphamide, Doxorubicin and Vincristine
cDC	conventional Dendritic Cell
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein diacetate Succinimidyl Ester
CIN	large-scale Chromosomal Instability
Con A	Concanavalin A
CM	Conditioned Medium
CXCL12	Chemokine (C-X-C motif) Ligand 12
CXCR4	C-X-C Chemokine Receptor Type 4
CT	Computed Tomography
Da	Daltons

DAB	3,3V-diaminobenzidine
DAG	Diacylglycerol
DAPI	4,6-diamidino-2-phenylindole
DC	Dendritic Cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
ED	Extensive-stage Disease
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene Glycol-bis (β-aminoethyl ether) N,N,N',N'-Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial–Mesenchymal Transition
FAK	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FcR	Fc Receptor
FGF	Fibroblast Growth Factor
FHIT	Fragile Histidine Triad
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead Box P3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GRP	Gastrin-Releasing Peptide
GRP-R	Gastrin-Releasing Peptide Receptor

GTPases	Guanosine Triphosphatases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte Growth Factor
HLA-DR	Human Leukocyte Antigen - antigen D Related
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon-γ
IGEPAL	Octylphenoxy poly(ethyleneoxy)ethanol
IGF1	Insulin-like Growth Factor 1
IHC	Immunohistochemistry
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IONO	Ionomycin
iTreg	induced Treg
L	Litre
LD	Limited-stage Disease
LDS	Lithium Dodecyl Sulfate
LOH	Loss of Heterozygosity
LPS	Lipopolysaccharides
m	milli
M	Molar (moles per L)
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MAPK	Mitogen-Activated Protein Kinase
MDM	Monocyte-Derived Macrophage
MDSC	Myeloid-Derived Suppressor Cell

MHC	Histocompatibility Complex
MLR	Mixed Leukocyte Reaction
MMP	Matrix metalloproteinase
MMR	DNA Mismatch Repair
mRNA	messenger Ribonucleic Acid
MRI	Magnetic Resonance Imaging
MSI	Microsatellite instability
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na	Sodium
NaCl	Sodium chloride
Na_3VO_4	Sodium orthovanadate
NK	Natural Killer
NKT	Natural Killer T
NMB	Neuromedin B
NMB-R	Neuromedin B Receptor
NOS	Nitric Oxide Synthase
NSCLC	Non Small Cell Lung Cancer
nTreg	naturally occurring Treg
PBMC	Peripheral Blood Mononuclear Cell
PBS	Dulbecco's Phosphate-Buffered Saline
pDC	plasmacytoid DC
PD-1	Programmed cell death- 1
PD-L1	Programmed cell death Ligand 1
PE	Cisplatin and Etoposide

PE	Phyccoerythrin (Flow Cytometry Fluorochromes)
PerCP	Peridinin Chlorophyll Protein Complex
PET	Positron Emission Tomography
PI3K	Phosphatidyl inositol-3 Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol Myristate Acetate
PMSF	Phenylmethylsulfonyl fluoride
RER	Replication Error Repair
RNA	Ribonucleic Acid
RPE	R-Phyccoerythrin
rpm	revolutions per minute
RTKs	Receptor Tyrosine Kinases
SCLC	Small Cell Lung Cancer
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
TAM	Tumour-Associated Macrophage
TBS	Tris-Buffered Saline
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-β
Th	T helper
TIL	Tumour-Infiltrating Lymphocyte
TNF	Tumour Necrosis Factor

TNM	T: primary tumour
	N: regional lymph nodes
	M: distant metastasis
Treg	Regulatory T cell
UK	United Kingdom
USA	United States of America
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel Lindau

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Chapter 1: Introduction

Chapter 1 Introduction

1.1 Lung Cancer

Lung cancer, a highly invasive, rapidly metastasizing and prevalent cancer, is the leading cause of cancer death in the UK and worldwide (1,2). It is broadly categorised into two forms of lung cancer according to the shapes of cells and clinical behaviour: non-small cell lung cancer (NSCLC) (about 85% of all lung cancer) and small cell lung cancer (SCLC) (about 15%) (1,3).

NSCLC can be further divided into three types. Adenocarcinoma accounts for 40% of lung cancer that occurs mainly in current or former smokers but is also the most common lung tumour seen in non-smokers. It arises in the epithelial cells of segmental bronchi and alveoli (1). Adenocarcinoma tumours grow slower than other types of lung cancer and are often found before metastasis (3). Squamous cell carcinoma is the second most common type of lung cancer (30-35%) and is closely correlated with a history of cigarette smoking (1). The tumour cells are derived from the epithelial cells of main and lobar bronchi, and usually located in within the lung (3). Large cell carcinoma (10%) derives from the hormonal cells of the lung cells (1). It tends to grow and spread quickly and therefore the prognosis and survival rate remain poor (4-6). Currently most patients with NSCLC are diagnosed at an advanced stage and have a poor prognosis (7). Despite advances in early detection and standard treatments including curative surgery or radiotherapy possibly in combination with chemotherapy, patient survival remains low. The mortality rate is much higher than other types of cancer and the predicted 5-year survival rate is 15.9% after diagnosis (7,8).

Small cell lung cancer (SCLC) is a highly malignant cancer (3). SCLC cells are composed of smaller than normal, undifferentiated, round oval or spindle-shaped cells. It often originates in proximal bronchi near the centre of the chest and it is characterised by rapid growth and early metastatic spread (9). SCLC has the most aggressive clinical course of any type of pulmonary tumour and thus surgical resection is rarely possible (10). SCLC is more responsive to chemotherapy and radiotherapy, however, SCLC has often widely disseminated by the time of diagnosis. The mortality from diagnosis is therefore greater than other types of lung cancer with median survival of 2-4 months. 2-year survival is less than 5% and 5-year survival is 5-10% for SCLC, despite treatment (10 -12).

Cigarette smoking is the greatest single risk factor for developing lung cancer (13). Other drivers of the lung cancer epidemic include radon exposure, atmospheric pollution, industrial exposure to carcinogens and chronic lung disease (13,14). It has been shown that SCLC displays a stronger relation with tobacco smoking than NSCLC, and over 95% of SCLC cases are attributable to cigarette smoking (15,16).

1.2 Clinical features, Diagnosis and Staging of SCLC.

Typically, SCLC grows silently and metastasizes to mediastinal lymph nodes early. Most patients with SCLC present with a short duration of symptoms, usually 8-12 weeks before presentation but have widespread disease at diagnosis (17). Symptoms can result from local tumour growth, intrathoracic

spread, distant spread, and/or paraneoplastic syndromes. These may include chest pain, cough, shortness of breath and hemoptysis. Moreover, the symptoms including headache, blurred vision, photophobia, nausea and vomiting are common (18). In addition to direct effects, SCLC is associated with paraneoplastic syndromes (19). These are triggered by an altered immune system response to a neoplasm or ectopic production of a hormone or cytokine, affecting the endocrine and neurologic systems in patients (19).

To assess SCLC at presentation and to guide its management, smoking history, blood tests, imaging (chest X-ray, CT, MRI and PET scan) are used, together with cellular phenotyping (sputum cytology and tissue biopsy analysis) (20,21). In some cases, it is difficult to differentiate from NSCLC based on morphology alone, therefore immunohistochemistry is also used in diagnosing SCLC (21).

Since SCLC is extremely chemo- and radiosensitive, systemic chemotherapy is presently the cornerstone of treatment of SCLC. The clinical purpose of staging is to decide whether thoracic radiation should be incorporated in combination with chemotherapy for localized disease. TNM (T: primary tumour, N: regional lymph nodes, M: distant metastasis) staging system is commonly used to stage NSCLC and most cancers (22). However, the clinical behaviours of NSCLC and SCLC are different and are treated in very different ways. The LD (limited stage disease) versus ED (extensive stage disease) classification system defined by the Veterans Administration Lung Study Group is widely utilized in SCLC because of its simplicity and clinical utility (23,24). LD is SCLC that is confined to one hemithorax including ipsilateral supraclavicular lymph nodes and can be

encompassed within a single radiation port. ED includes any cases of SCLC that have spread to the opposite lung and/or to distant sites in the body (23).

1.3 Treatment of SCLC.

Since SCLC is typically disseminated or at least locally advanced and 60-70% patients have ED at the time of diagnosis, the possibility of surgical resection immediately is unlikely (25). However, SCLC cells are extremely sensitive to chemotherapeutic drugs in the primary treatment modality (26). Therefore, the typically treatment of SCLC is chemotherapy with or without radiation. However, despite initial objective response rates, tumour relapses are common and most patients eventually succumb to the disease (27).

1.3.1 Chemotherapy

The discovery of chemotherapy as a treatment for SCLC has significantly improved patient survival. The commonly used combination chemotherapeutic agents to treat SCLC include cyclophosphamide (C), doxorubicin (A), and vincristine (V), (CAV); cyclophosphamide (C), doxorubicin (A), and etoposide (E), (CAE); and cisplatin (P) and etoposide (E), (PE) (28). Novel agents with significant single-agent activity in SCLC, include paclitaxel, docetaxel, vinorelbine, irinotecan and topotecan and gemcitabine (28). The standard chemotherapy for the first-line treatment of SCLC is 4-6 cycles of PE (29,30). The combination of carboplatin and etoposide has been shown to have similar effect as PE in both LD and ED patients, the median survival is approximately 12 months (31). A number of studies have also demonstrated significant survival

advantage with the irinotecan-cisplatin regime compared with PE in the patients with ED. Median survival has been reported to have improved from 9.4 months to 12.8 months, and 2-year-survival from 5.2% to 19.5% (32-35). Notably, however, a further phase III trial in America has failed to confirm this result (36). This may be because the response duration is often short (approximately 9 months), the tumour becomes resistant to the chemotherapy (37). Following tumour relapse, second-line chemotherapy is much less effective than initial treatment (38). A number of studies have attempted to address this by intensifying chemotherapy dose, using new combinations of chemotherapeutic agents and trailing new drugs to prolong patient survival (10).

1.3.2 Radiotherapy

In addition to cytotoxic therapy, SCLC cells are more sensitive to radiotherapy than NSCLC (39). Meta-analyses have shown that the addition of thoracic radiation therapy to chemotherapy moderately improves the survival in patients with LD SCLC (40, 41). A dose of 45 Gray (Gy) of concurrent thoracic radiotherapy is considered as standard therapy. Earlier (less than 30 days) chest radiotherapy with platinum-based chemotherapy improved 2- and 5-year survival in LD SCLC patients (42). Furthermore, patients given twice daily irradiation showed significantly improved survival rates in LD SCLC (43).

1.4 Pathogenesis of SCLC.

The pathogenesis of SCLC is like other cancers, beginning with carcinogen-induced initiation events, followed by a long period of promotion and progression

in a multistep process. The process involves malignant transformation, uncontrolled cellular proliferation, avoidance of apoptosis, angiogenesis, migration and invasion of distant tissues.

1.4.1 Carcinogenesis

SCLC, similar to other lung cancers, is the end stage of multiple-step carcinogenesis, driven by genetic and epigenetic damage caused by chronic exposure to tobacco smoke carcinogens. More than 20 carcinogens contained in tobacco smoke are strongly associated with development of lung cancer (44). These include polycyclic hydrocarbons, nitrosamines and aromatic amines. Chronic exposure to such agents induces DNA damage, misreplication and mutation and so smokers are predisposed to developing lung cancer (45, 46). The specific sequence of genetic instability in SCLC remains unclear. However it has been shown to exist at two levels. Large-scale chromosomal instability refers to losses or gains of whole or large portions of chromosomes. Loss of heterozygosity (LOH) refers to loss of normal, functional alleles at a heterozygous locus. It may arise from several mechanisms (47). LOH on chromosome 3p affecting multiple genes is one of the most frequent and earliest genetic alterations in SCLC pathogenesis and is observed in more than 90% of SCLC (48). These affected regions contain a variety of tumour suppressor genes such as fragile histidine triad (FHIT) and von Hippel Lindau (VHL), and may therefore contribute to the development of SCLC (49,50). Other common chromosomal alterations include loss of 5q, 17p and 13q, which are loci with tumour suppressor genes including *p53* and *Rb* (51). In addition, comparative genomic hybridization analyses have revealed that a large number of SCLCs

harbor gains of 1p, 2p, 3q, 5p, 8q and 19p. These regions encode well-known proto-oncogenes, such as *MYC* and *KRAS* (52). Microsatellite instability (MSI) refers to small deletions or expansions in the length of repetitive DNA microsatellites, which consists of long, tandem repeats of between one and six nucleotides (53). Replication error (RER) leading to MSI has been implicated in the pathogenesis of tumour (53). RER derived from deficiency or mutations in DNA mismatch repair (MMR) genes has been found in approximately 35% of SCLC (54).

1.4.2 Dysregulated Cellular Proliferation

A variety of peptide growth factors and their receptors are secreted and expressed by SCLC cells and adjacent normal cells (55). It leads to the development of several autocrine (secreted by and act on the same cell) and paracrine (secreted by one cell and act on another) growth stimulatory loops (55). The best-characterized autocrine system in SCLC includes three bombesin-like peptides (BLPs) and their receptors. Bombesin is a 14-amino-acid neuropeptide (56), Gastrin-releasing peptide (GRP) is a 27-amino-acid mammalian homologue of bombesin, Neuromedin B (NMB), a 10-amino-acid peptide, is the third member of BLP family (56). Bombesin and GRP share a conserved bioactive C-terminal sequence so that both peptides have the same physiological effects (57). They signal through G protein-coupled BLP receptors: GRP receptor (GRP-R), NMB receptor (NMB-R), and bombesin receptor subtype 3 (BB3) (56, 58). GRP-R has high affinity for bombesin and GRP and very low affinity for NMB (58). Through the binding of GRP-R, multiple cellular signal transduction pathways are activated (Fig. 1.1), including tyrosine

phosphorylation of focal adhesion kinase (FAK) and activation of protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinases (PI3Ks) (59). These activated signaling pathways mediate cell proliferation, survival and migration during umour progression.

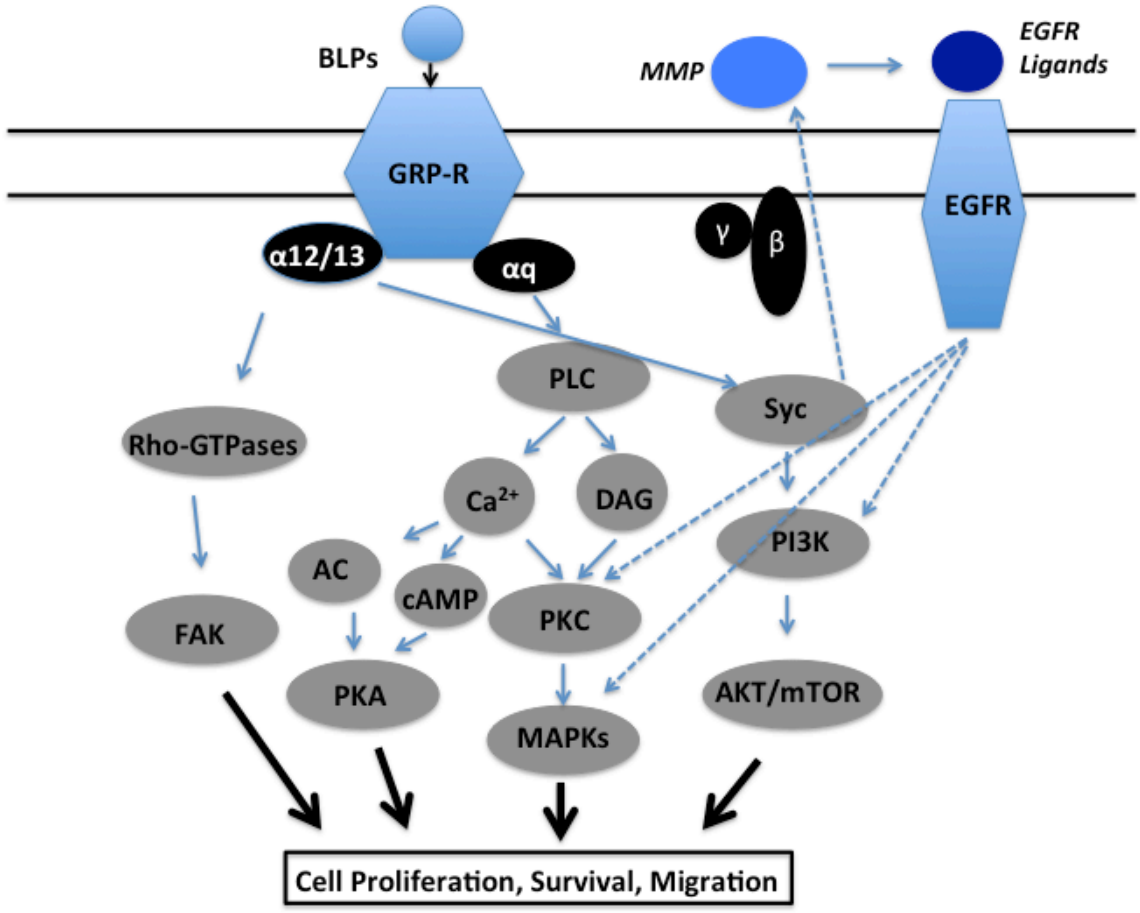


Figure 1.1: Signaling pathways associated with GRP-R in cancer. The GRP-R is a G protein-coupled receptor, after agonist binding, the receptor expose intracellular sites involved in the interaction with G-protein heterotrimer, which contains α, β and γ subunits. GRP-R interacts with Gαq and Gα12/13 subfamilies. Gαq activation triggers phospholipase C (PLC)-mediated generation of the second messengers, which induce rapid release of Ca^{2+} and diacylglycerol (DAG) that activate protein kinase C (PKC). The activation of PKC, in turn, activates mitogen-activated protein kinase (MAPK) pathways. Ca^{2+} release can also induce adenylyl cyclase (AC) and cyclic adenosine monophosphate (cAMP), leading to activation of protein kinase A (PKA). In addition, Gα12/13 subunit of G protein activates the Rho family of guanosine triphosphatases

(GTPases), which induce tyrosine phosphorylation of a set of focal adhesion kinases (FAKs). The phosphorylation of Src (the largest family of non-receptor protein tyrosine kinases) stimulated by Gα12/13 leads to downstream induction of phosphatidylinositol 3-kinase (PI3K) and subsequent activation of Akt and mTOR. Src can also be activated by βγ subunits, mediate matrix metalloproteinase (MMP) activity and release of epidermal growth factor receptor (EGFR) ligands. The subsequent phosphorylation of EGFR leads to activation of PKC and MAPK signalling pathways. These cell signalling pathways associated with GRP-R regulate tumour cell proliferation, survival and migration.

Up to 60% of SCLC cell lines express GRP and all express NMB (60,61). The mechanism by which this growth stimulatory autocrine loop remains unclear. Mutations of either GRP/bombesin or their receptor have not been identified in lung cancers (62). However, GRP-R is transcriptionally up-regulated with long-term tobacco exposure. GRP-R activation is associated with proliferative response of bronchial cells *in vitro* to the mitogenic effects of BLPs (63). Blocking GRP-R signaling with neutralizing antibodies against GRP/bombesin or receptor antagonists inhibits tumour growth *in vivo* and *in vitro* (64). The development of resistance to conventional chemotherapy, which is inevitable in SCLC, is accompanied by an increase in expression of neuropeptide growth factor receptors (65). These findings support a role for BLP signalling in the pathogenesis of SCLC.

SCLC cells also display dysregulated expression of other crucial cell proliferation regulators. *Myc (myc, N-myc and L-myc)* family genes are involved in a wide range of cellular processes including proliferation, differentiation and tumourigenesis (67). Expression and amplification of these genes has been found in about 20% SCLC tumour samples and 50% of SCLC cell lines (66,67).

Moreover, increased N-Myc expression has been correlated with poor survival and chemotherapy response in SCLC (68). Inactivation of tumour suppressor *p53* and *Rb* genes, which are also involved in the induction of apoptosis and control of cell cycle, occur commonly in SCLC (69).

1.4.3 Avoidance of Apoptosis

A reduced tendency to programmed cell death (apoptosis) is a crucial step in malignant transformation and is also important in resistance to chemo and radiotherapies. Over expression of the *BCL-2* proto-oncogene that protects cells from apoptosis is common in SCLC tumours and cell lines, and may thus play a role in the pathogenesis of SCLC (70, 71). In addition, SCLC cells frequently display mutations of the *p53* gene, which may cause both loss of its tumour suppressor functions and loss of the ability to induce apoptosis, cell cycle arrest and DNA repair (69, 72). Moreover, restoration of p53 function is sufficient to suppress the growth of SCLC cells due to induction of apoptosis (69). Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases that regulate several cellular processes including proliferation, growth, apoptosis, and cytoskeletal rearrangement (73), and the PI3K/Akt signaling pathway delivers an anti-apoptotic signal to promote cell survival (74). These proteins are constitutively active in SCLC cell lines. PI3K inhibition markedly inhibits SCLC cell proliferation in an anchorage-independent fashion and promotes apoptosis (75). In addition, in a subset of SCLC where a PI3KCA mutation is present, inhibition of PI3K signalling induces apoptosis and inhibits tumour growth in SCLC (76).

1.4.4 Angiogenesis

Angiogenesis is crucial in neoplastic development and progression. The formation of new blood vessels not only supplies oxygen and nutrients, but also provides a means for the tumour to spread haematogenously (77). Vascular endothelial growth factor (VEGF) plays a critical role in angiogenesis (78). Serum VEGF level is significantly higher in SCLC patients and correlates with poor survival (79). Inhibition of the VEGF receptor blocks SCLC cell proliferation and angiogenesis *in vivo* (80), and over-expression of VEGF on tumour biopsies predicts poor prognosis in SCLC patients (81). In addition, increased levels of basic fibroblast growth factor (bFGF), another growth factor of angiogenesis, is observed in serum from SCLC patients and correlates with survival (82).

1.4.5 Migration and Invasion

Whilst SCLC is often disseminated at the time of clinical presentation, the mechanisms by which SCLC cells invade tissue remain unclear. Many receptor tyrosine kinases (RTKs) are overexpressed in SCLC, notably c-Met (83). Stimulation of SCLC cells with hepatocyte growth factor (HGF) (the ligand for c-Met) significantly increases formation of filopodia and migration of cellular clusters (84). Matrix metalloproteinases (MMPs), a family of zinc-containing proteolytic enzymes, facilitate tumour invasion, the establishment of metastases, and the promotion of tumor-related angiogenesis (85). MMPs (MMP-1, -2, -7, -9, -14 and -15) are widely expressed in SCLC and represent independent negative prognostic factors for survival (86). E2F1, a transcription factor for MMPs is overexpressed and represents an independent and adverse prognostic factor. Depletion of E2F1 inhibits SCLC cell migration and invasion (87). SCLC has a

propensity for metastasis to the bone marrow (88). C-X-C chemokine receptor type 4 (CXCR4, CD184) acts as a receptor for chemokine (C-X-C motif) ligand 12 (CXCL12) is highly expressed on SCLC cells (89). CXCR4 has a central role in haematopoetic cell migration to the bone marrow, and so CXCR4: CXCL12 signaling may stimulate SCLC cell invasion through extracellular matrix and bone marrow metastasis (89).

1.5 Tumour Microenvironment.

The tumour microenvironment is the cellular environment in which the tumour exists. Stromal elements include surrounding blood and lymph vessels, fibroblasts of various phenotypes, extracellular matrix (ECM) and many different types of immune and inflammatory cells (90). The interactions between malignant and non-transformed cells can contribute to cancer progression, invasion and metastasis (90).

1.5.1 Extracellular matrix (ECM)

The ECM is a large collection of extracellular molecules secreted by resident cells, including proteins, glycoproteins, proteoglycans and polysaccharides with different physical and biochemical properties (91). The function of ECM is to regulate cell adhesion, migration, cell-to-cell communication and differentiation. Conversely, abnormal ECM contributes to cancer progression by promoting cellular transformation and metastasis as well as facilitating tumour-associated angiogenesis and inflammation (92). In SCLC, it has been shown that ECM proteins support SCLC cell growth *in vivo* (93) and SCLC tumours are

extensively infiltrated by ECM proteins (fibronectin, laminin and collagen IV) at both primary and metastatic sites (94). In addition, adhesion of SCLC cells to ECM *in vitro* enhances tumorigenicity and confers resistance to apoptosis induced by chemotherapeutic agents through interactions with b1 integrins (α2β1, α3β1, α6β1 and αvβ1) (94).

1.5.2 Fibroblasts

Fibroblasts are flat, elongated non-differentiated cells in the connective tissue. The functions of fibroblasts include deposition of ECM, regulation of epithelial differentiation, regulation of inflammation and involvement in would healing. (95,96). Myofibroblasts are a subpopulation of fibroblasts that are differentiated from residential fibroblasts in response to paracrine signals (97). Activated myofibroblasts expressing α-smooth-muscle actin within desmoplastic lesions are associated with malignant tumours and called cancer-associated fibroblasts (CAFs) (98). There is increasing evidence showing that CAFs are important promoters of tumour growth and progression (99). Various growth factors secreted by CAFs including HGF, fibroblast growth factor (FGF) and insulin-like growth factor 1 (IGF1) are mitogenic for malignant cells (90). Moreover, fibroblasts are an important source of MMPs and CXCL12 that can favor tumour growth, invasion and metastasis (99,100). In addition, SCLC cell lines secrete a variety of molecules that are stimulatory to fibroblasts, including GRP and transforming growth factor-β (TGF-β) (101, 102). GRP promotes fibroblast proliferation (103). TGF-β can increase ECM production (104), induce epithelial–mesenchymal transition (EMT) in malignant cells and promote immunosuppression (105).

1.5.3 Immune cells and the tumour microenvironment

In addition to fibroblasts, tumour stroma contains various cells of the immune system (Fig. 1.2). In SCLC, the local immune cell infiltrates have not been extensively studied. In other types of cancers, such infiltrates include tumour-infiltrating lymphocytes (TILs): T cells and B cells, natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells (DCs) and macrophages. These cells are capable of driving potent anti-tumour responses. However, in many cancers, tumour cells can induce a tumour-tolerant microenvironment by immunomodulatory mechanisms (90). Such effects may support cancer growth and spread (106,107).

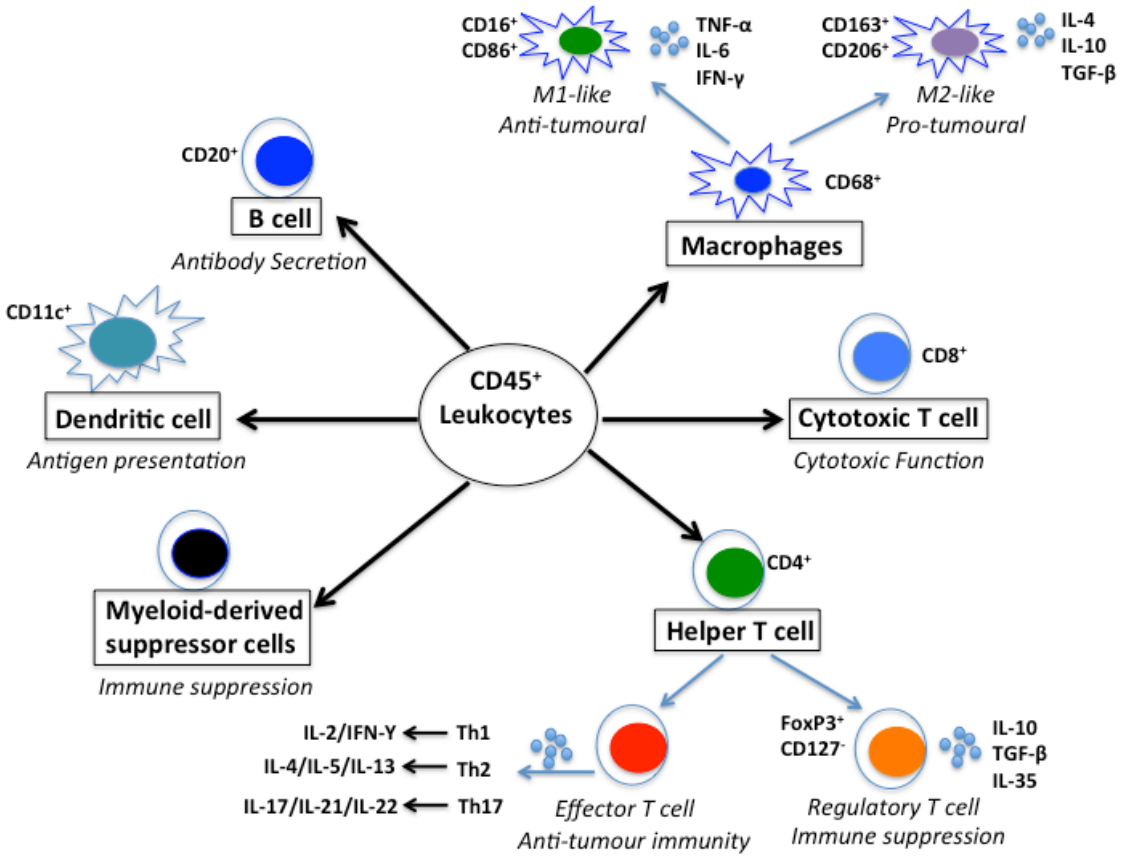


Figure 1.2. Immune cells within the tumour microenvironment. $CD45^+$ leukocytes that infiltrate into tumour comprise diverse subsets of immune cells: lymphocytes ($CD3^+$) include T cells and B cells. B cells ($CD20^+$) function to capture antigens and release antibodies. T cells include $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. Effector $CD4^+$ T cells play a central role in anti-

tumour immune response and can be further divided into different subsets according to their functions and cytokine production (Th1, Th2, Th17). Regulatory $CD4^+$ T cells are immunosuppressive, characterized by the high level of FoxP3 expression, low level of CD127 expression and anti-inflammatory cytokine release. Dendritic cells ($CD11c^+$) function as potent antigen presenting cells. Myeloid-derived suppressor cells function to inhibit immune cell activation. Macrophages ($CD68^+$) within tumour display different phenotypes and functions during tumour progression: Anti-tumoural M1-like phenotype with high levels of CD16 and CD86 expression and pro-inflammatory cytokine production; Pro-tumoural M2-like phenotype with high levels of CD163 and CD206 expression and anti-inflammatory cytokine production.

1.5.3.1 T lymphocytes

Elevated levels of TILs are associated with better prognosis in many cancers (108). $CD8^+$ cytotoxic effector T cells kill tumour cells by releasing perforin and granzymes to induce apoptosis, and are strongly associated with a good prognosis (109). $CD4^+$ T helper 1 (Th1) cells favor cellular immune responses by producing the cytokines interleukin-2 (IL-2) and interferon gamma (IFN-γ), and by interacting with $CD8^+$ cytotoxic T cells, NK cells and macrophages (109). $CD4^+$ T helper 2 (Th2) cells act on B cells, favour humoral immunity by producing IL-4, IL-5 and IL-13. $CD4^+$ T helper 17 (Th17) cells favour anti-microbial tissue inflammation by producing IL-17A, IL-17F, IL-21 and IL-22, they are generally thought to promote tumour growth (109). $CD4^+$ regulatory T (Treg) cells play immunosuppressive effects in a variety of immune cells, and high numbers of these cells in the tumour microenvironment correlate with worse prognosis in many cancers (110-112). In addition, γδ T cells present in the tumour microenvironment play cytotoxic activity against a wide range of tumour cells by releasing large amounts of IFN-γ and tumour necrosis factor (TNF)-α (113).

1.5.3.2 $CD4^+$ Treg cells

$CD4^+$ Treg cell infiltration has been described in human NSCLC and ovarian cancer (114). These cells are characterized by the expression of forkhead box P3 (FoxP3) and CD25 (115). Treg cells function beneficially *in vivo* to prevent autoimmunity (116, 117). However, their infiltration into tumour stroma raises the possibility that their recruitment may be a mechanism whereby $CD4^+$ and $CD8^+$ effector T cell mediated anti-tumour immune responses are abrogated (118-121). A preponderance of $CD4^+$ Treg cells may lead to a failure of tumour immunosurveillance and contribute to the progression of cancer. Two general subpopulations of Treg cells have been classified based on FoxP3 expression (Fig. 1.3). Both inhibit recognition and clearance of tumour cells by the immune system. Naturally occurring Treg (nTreg) cells are derived from the thymus. Adaptively induced Treg (iTreg) cells differentiate from resting $CD4^+$ T cells as part of the response to antigen challenge (122). The mechanisms by which Treg cells suppress immune responses have been described (122). These include physical elimination of cytotoxic cells by direct cell-to-cell contact, secretion of potent immunosuppressive cytokines IL-10 and TGF-β, and inhibition of proliferation and/or cytokine production in pathogenic T cells (122). In addition, Treg cells can indirectly suppress effector T cell activation via inhibition of the stimulatory capacity of antigen presenting cells (APCs) (122).

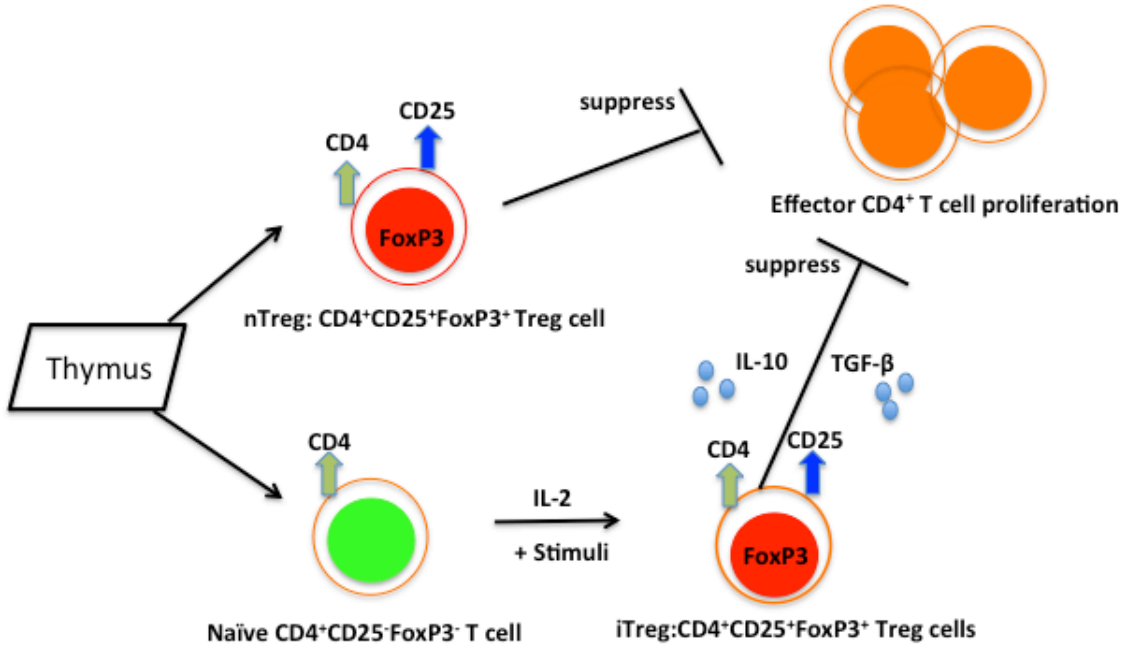


Figure 1.3: $CD4^+$ Treg cell subpopulations. Naturally occurring Treg (nTreg) cells ($CD4^+CD25^+FoxP3^+$) and naïve T cells ($CD4^+CD25^-FoxP3^-$) are derived from the thymus. nTreg cells function to suppress effector $CD4^+$ T cell proliferation. Upon activation, naïve $CD4^+$ T cells can be induced to differentiate to adaptively induced Treg (iTreg) cells in the periphery. These iTregs have the same phenotype $CD4^+CD25^+FoxP3^+$ and suppressive function. Their suppression mechanisms are mediated through secreting immunosuppressive cytokines IL-10 and TGF-β.

There are also another two populations of iTregs developed from resting $CD4^+CD25^-$ T cells that do not express FoxP3: Tr1 (123) and Th3 (124) cells. Tr1 cells are defined by their ability to produce large amounts of IL-10 and low levels of TGF-β, whereas Th3 cells produce preferentially TGF-β. It has been shown that IL-10-producting Tregs can be induced by stimulation of resting $CD4^+$ T cells in the presence of CD46 (the complement regulator protein) under homeostatic conditions (125). Tr1 cells inhibit activation of naïve and memory T cells and suppress Th-1 and Th-2-mediated immune responses to pathogens

and tumours, and their suppressive effects are mainly mediated through IL-10 production (126). TGF-β is important for generation of Th3 cells, and their suppressive effects are antigen non-specific and mediated through secretion of TGF-β (124).

Increased frequency of Treg cells in TILs compared to their prevalence in peripheral blood has been found in colorectal, gastric and esophageal cancer patients (127,128). Furthermore, the proportion of circulating Treg cells in the peripheral blood of cancer patients is much higher than healthy individuals (129-131).

1.5.3.3 NK and NKT cells

Innate cytotoxic lymphocytes, NK and NKT cells are present in the tumour microenvironment. The important functions of NK cells are to distinguish stressed cells (infected cells, tumour cells and cells that undergone physical or chemical injuries) from healthy cells, and to secrete cytokines (such as IFN-γ) to initiate adaptive immune response (132). NK cell infiltration appears to predict good prognosis in many cancers such as colorectal (133), gastric (134), lung (135), renal (136) and liver (137). NKT cells possess characteristics of both T and NK cells and play an active role in tumour immunosurveillance (132). The increased infiltration of $V\alpha24^+$ NKT cells has been observed to be an independent prognostic factor for favorable prognosis in colorectal carcinomas (138). Conversely, a number of studies suggest that NK cells in the tumour microenvironment have an anergic phenotype that is induced by malignant cell-

derived TGF-β (109).

1.5.3.4 Myeloid-derived suppressor cells (MDSCs)

MDSCs are defined as a heterogeneous population of polymorphonuclear and monocytic $CD11b^{+}GR1^{+}$ cells that inhibit the activation of immune cells (139). They originate from haematopoietic stem cells and differentiate into various cells, including monocytes/macrophages, DCs and granulocytes, depending on the types and stages of tumour and physiological conditions (139,140). MDSCs can suppress $CD8^{+}$ T cell activation through the expression of nitric oxide synthase 2 (NOS2) and arginase (ARG1) (141). In addition, they induce Treg cell development through secreting IL-10 and down-regulating IL-12 (142), and induce polarization of alternatively activated M2 macrophages through cell-contact interaction (143).

1.5.3.5 Tumour-associated macrophages (TAMs)

Macrophages can infiltrate both primary and secondary tumours, where they exhibit a distinct phenotype. These cells are termed tumour-associated macrophages (TAMs) (144). TAMs present within the solid tumours display a tumour-promoting phenotype with various functions in the development of tumours (145, 146). These functions include stimulation of tumour cell proliferation and angiogenesis, promotion of malignant cell migration, invasion and metastasis, and suppression of anti-tumour immunity (146). The functions of TAMs are shown below in Figure 1.4 (146, 147).

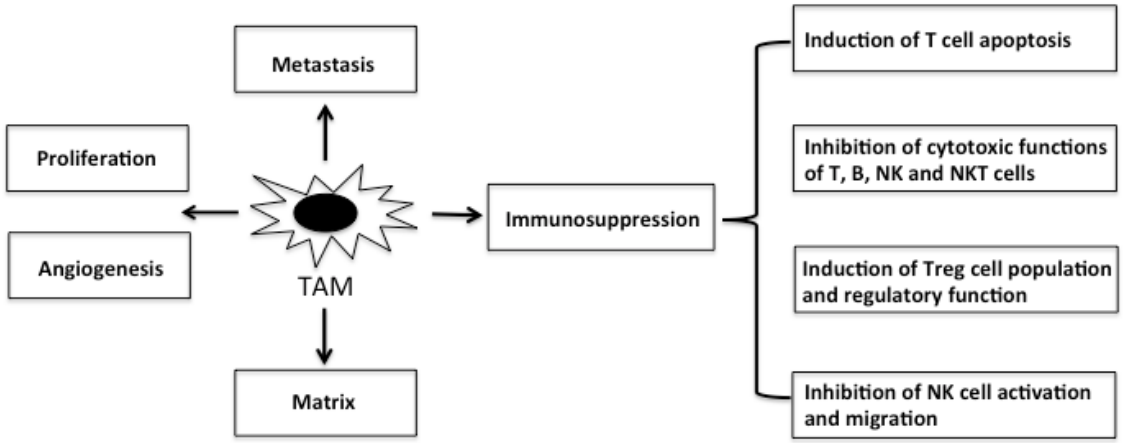


Figure 1.4: The functions of TAMs in the tumour development. These functions include stimulation of tumour cell proliferation and angiogenesis, promotion of malignant cell migration, invasion and metastasis, and suppression of anti-tumour immune responses. The mechanisms of immunosuppression include induction of T cell apoptosis, inhibition of cytotoxic functions of T cells, B cells, NK and NKT cells, induction of Treg cell population and regulatory function, and inhibition of NK cell activation and migration.

The mechanisms of immunosuppression by TAMs include induction of T cell apoptosis, inhibition of cytotoxic functions of T cells, B cells, NK and NKT cells, induction of Treg cell population and regulatory function, and inhibition of NK cell activation and migration (Fig. 1.4) (147). The relationship between the TAM population within tumour microenvironment and cancer patient survival may be complex. Pre-clinical and clinical evidence shows that a high density of TAM infiltration is associated with poor prognosis in various cancers, including breast, cervix, prostate, bladder, kidney, squamous cell carcinoma and follicular lymphoma (144,148). However, in contrast, they are associated with better prognosis in melanoma, stomach, lung and brain tumours (148).

Macrophages can be broadly divided into two types: classically activated (M1) and alternatively activated (M2) macrophages (144, 146). M1 macrophages tend

to display anti-tumour effects. They are efficient immune effector cells, capable of killing pathogens and tumor cells, presenting antigens, and producing high levels of pro-inflammatory cytokines (IL-1, IL-6, and TNF-α). M2 macrophages tend to have pro-tumoral effects. They have poor antigen-presenting ability, are capable of suppressing T cell proliferation and function, and secrete high levels of anti-inflammatory cytokines (IL-4, IL-10, IL-13 and TGF-β) (144, 146). The phenotype and function of TAMs during tumour initiation differ from that during tumour progression. This may be compared to a switch from an M1-like to an M2-like phenotype. TAM polarization is dependent on the signals they receive from the particular tumour microenvironment in which they reside (149).

1.5.3.6 Dendritic cells (DCs)

Dendritic cells (DCs) are professional APCs of the host immune system. They play important roles in antigen processing and presentation, and T cell stimulation. DCs can be recognized by two major subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) according to the morphology, marker and function (150). A number of studies have demonstrated decreased presence and defective functionality of mature DCs in various cancer patients. Their abilities of tumour-associated antigen processing and T cell immune response stimulation are impaired (151-156). In addition, DCs at tumour sites have been shown to secrete TGF-β and stimulate Treg cell proliferation (157,158). They may further suppress effector T cell immune function against tumours (159).

1.6 Immune Response in SCLC

The immune system protects the host from tumour development, growth and metastasis. However, most patients with SCLC demonstrate several important local and systemic immune defects that correlate with worse morbidity and mortality (160,161). Delayed hypersensitivity skin test reactivity and a decreased CD4/CD8 ratio in peripheral blood lymphocytes has been observed to correlate with poor survival in SCLC patients (160, 161). Peripheral blood lymphocytes from SCLC patients show significantly reduced proliferative responses to phytohemagglutinin and impaired secretion of IL-2 and macrophage-activating factor (162). Furthermore, decrease of IL-2 secretion correlates with poor survival in SCLC patients (163). Histological analysis of lung cancer sections demonstrates that the amount of cellular infiltration of inflammatory cells is lowest in SCLC compared with other lung tumours (164). Proliferating lymphocytes isolated from tissue fragments from SCLC biopsies cultured in IL-2 are ineffective in lysing autologous tumour cells, though lymphocytes from NSCLC patients are effective (165). Alveolar macrophages (AM) from SCLC patients have shown impaired phagocytic function, decreased production of inflammatory cytokines (TNF-a, IL-1 and IL-6) (166). In addition, expression of major histocompatibility complex (MHC) class II antigen and intercellular adhesion molecule 1 (ICAM-1) and CD83 is reduced (166).

MHC class I is required for tumour antigen presentation and also subsequent cytotoxic T cell-mediated tumour cell lysis (167). However, the expression of MHC class I antigens is markedly decreased on SCLC cells and in SCLC

tumour sections (168). Thus down-regulation of MHC class I may represent an important mechanism for the development of SCLC in the early stage. The programmed cell death- 1 (PD-1) receptor is an immunoinhibitory receptor that belongs to the CD28 family. It is expressed on T cells, B cells, monocytes, NK cells and many TILs (169). The interaction of PD-1 and its ligand PD-L1 inhibits T lymphocyte proliferation, survival and effector functions, induces apoptosis of tumour-specific T cells and promotes the differentiation of $FoxP3^+$ Treg cells (170). Cancer cells may escape immunosurveillance by expressing PD-L1 to down-regulate immune response. Therefore blockade of immune checkpoints with monoclonal antibodies has been recently developed as a new therapeutic strategy to improve immunity in cancers. However, in SCLC, the PD-L1 expression is present in over 70% of tumour cells and positively correlated with patient survival (171). It may indicate that the expression of PD-L1 by SCLC tumour cells highlights the existence of the anti-tumour response, the patients with negative PD-L1 expression may have poor immune response and lead to worse survival in SCLC. Therefore, the interaction between PD-L1 expression and the host immune responses within the tumour microenvironment in SCLC patients, may differ from other tumours and need to be further studied. Importantly, serum from SCLC patients inhibits antibody synthesis and expression of T cell membrane markers (172). Similarly, treatment with supernatant from SCLC cell lines can suppress peripheral blood lymphocyte proliferation in response to phytohemagglutinin (173). These observations indicate that SCLC cells are capable of producing soluble immunosuppressive factors, which may account, at least in part, for immune deficiency in SCLC patients.

1.7 Project Aims

The host immune system is now becoming an attractive target for the development of novel anti-cancer therapy. In SCLC, most patients are immunocompromised with poorly understood local and systemic immune defects that correlate with worse morbidity and mortality. Previous studies suggest that SCLC tumour cells may specifically suppress cell-mediated immune responses. Further understanding the mechanisms of immune suppression mediated by SCLC cells is required before the development of effective immune therapy. $CD4^+$ T cells play a central role in coordinating anti-tumour immune responses (174). $FoxP3^+$ Treg cells are crucial in mediating suppression of anti-tumour immune responses and present at high frequencies in tumour tissues of various types of cancers (175,176). Macrophages are particular abundant in tumour tissues and present at all stages of tumour progression with different functions (146). The presence and effects of these immune cells on tumour growth have not been studied in SCLC. In this thesis, I investigate the interactions between tumour cells and these immune cells to understand mechanisms by which SCLC cells mediate immune suppression.

SCLC has a very poor prognosis and only a small number of clinical markers are currently useful to stratify patients with SCLC into prognostic groups (177). Immune cells in the tumour microenvironment can eradicate malignant cells and control tumour growth. In SCLC, their analysis may therefore reveal novel prognostic markers to predict patient survival. This may assist medical decision making, improve future research study design and identify potential therapeutic

targets. However, the role of local immune cell infiltrates and specifically the impact of mononuclear cell and Treg cell infiltrates has not been extensively studied in SCLC. In this thesis, I study immune cells within the tumour microenvironment in biopsy samples from SCLC patients and correlate this with patient survival.

1.7.1 Hypothesis:

The body of work in this thesis addresses the hypotheses that:

SCLC cells mediate immunosuppression through modulating the host immune cell responses. Relatedly, the infiltration of immune cells within the tumour microenvironment correlates with patient survival in SCLC.

1.7.2 Specific Aims:

The overall aims of this work were therefore to determine further mechanisms of immunosuppression by SCLC cells and to identify a prognostic biomarker to predict patient survival in SCLC.

The following specific aims were:

1) To determine the mechanisms by which cell lines derived from SCLC tumours can suppress anti-tumour immune response *in vitro* by investigating:

    a) Whether and how SCLC cells suppress cell-mediated immune response;
    b) Whether SCLC cells induce Treg cell population, and identify the phenotype and function of these cells;
    c) Whether SCLC cells can affect macrophage polarization.

2) To assess local immune cell infiltrates into tumour tissues and to correlate this with patient survival in SCLC by:

    a) Identifying the population and location of immune cell infiltrates within SCLC biopsies;
    b) Investigating whether infiltration of Treg cells ($FoxP3^+$) and/or mononuclear leukocytes ($CD45^+$) have predictive effects on SCLC patient survival.

Chapter 2: Materials and Methods

Chapter 2 Materials and Methods

2.1 Cell Lines and Maintenance Cultures

Mycoplasma-free small cell lung cancer cell lines, NCI-H69, NCI-H345 were purchased from ECACC (Health Protection Agency, Porton Down, UK) and NCI-H510 from ATCC (LGC Standards, Teddington, UK). The cells were maintained in in HEPES buffered RPMI 1640 medium (Gibco®, Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 5 µg/ml L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin (Gibco®, Thermo Fisher Scientific). SCLC cells were cultured in suspension at 2 - 5 x 10^5 cells/ml and passaged up to 10 times prior to use in 75cm^2 flasks (Greiner Bio One, UK) at 37^oC in a humidified 5% CO_2 incubator.

2.2 SCLC cell Conditioned Medium (CM) and Cell Lysate

H69 and H510 cells grown as above were washed with Dulbecco's phosphate-buffered saline (PBS) without calcium/magnesium (Ca^{2+} and Mg^{2+} free) (Gibco®, Thermo Fisher Scientific) and resuspended at 2.5 x 10^6/ml in 10 ml Iscove's Modified Dulbecco's Medium (IMDM) (Gibco®, Thermo Fisher Scientific) supplemented with 50 U/ml penicillin and 50µg/ml streptomycin, but without FBS, in 25 cm^2 tissue culture flasks (Greiner Bio One, UK) for 72 hours. In some experiments, anti-IL-15 neutralising antibody (200 ng/ml, eBioscience), anti-TGF-β neutralising antibody (10 µg/ml, R&D Systems), or isotype control antibody was added to H69 cell culture and incubated for 72 hours. The serum-free conditioned medium (CM) was centrifuged at 300g for 5 minutes to remove cells and collected prior to use in section 2.13.

To lyse cells, the cell pellet was resuspended in lysis buffer ((10X Cell Lysis Buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin， Cell Signaling Technology, UK) was diluted to 1X solution in ddH_2O with 1mM Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, UK), 1X MINI protease inhibitor cocktail tablet (Roche)), and incubated for 30 minutes on ice. The cell lysates were centrifuged (14,000g) for 10 minutes at 4^{o}C and supernatant was collected to use in section 2.15.

2.3 Isolation of peripheral blood mononuclear cells (PBMCs)

Human peripheral blood was obtained from normal healthy donors approved by Guy's Hospital Ethics Committee, King's College London, and full written informed consent was obtained from all subjects. The peripheral blood was layered onto Lymphoprep^{TM} (Axis-Shield, Cambridgeshire, UK). PBMCs were isolated by density gradient centrifugation at 2,000g for 20 minutes. In brief, the blood was diluted with sterile PBS (Ca^{2+} and Mg^{2+} free) at ratio 1:1. 15 ml of room temperature Lymphoprep^{TM} was added into 50ml Falcon^{TM} (Greiner Bio One, UK) tubes. 25 ml of diluted blood was carefully layered onto Lymphoprep^{TM} in each tube using a sterile pastette and centrifuged at 2,000g for 20 minutes with no brake. The white cells from the interface were removed with a sterile pastette and washed three times with PBS by centrifugation at 1000g for 5 minutes to remove platelets. The cells were resuspended in complete IMDM (10% FBS, 50U/ml penicillin and 50µg/ml streptomycin) prior to use in sections 2.4, 2.5 and 2.7.

2.4 Isolation of $CD4^+$ T cells and naïve subset

Purity of cell isolates was evaluated by flow cytometry. $CD3^+CD4^+$ cells were isolated to >95% purity from PBMCs by negative selection using $CD4^+$ T cell isolation kit (Miltenyi Biotec, UK) according to the manufacturer's instructions. Naïve $CD4^+CD45RA^+$ T cells were isolated to this purity from PBMCs using naïve T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. In brief, PBMCs were resuspended in 40 µl MACS buffer (PBS Ca^{2+} and Mg^{2+} free, with 0.5% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.2, sterile filtered) per 10^7 cells and incubated with 10 µl of naïve/$CD4^+$ T cell Biotin-Antibody Cocktail per 10^7 cells for 5 minutes at 4^oC. A further 30 µl of MACS buffer and 20 µl of anti-biotin naïve/$CD4^+$ T cell anti-biotin MicroBead Cocktail were added per 10^7 cells and incubated for 10 minutes at 4^oC. The cell suspension was separated with LS columns (Miltenyi Biotec) placed in the magnetic field of a suitable MACS separator. The flow through of unlabelled cells (enriched naïve/$CD4^+$ T cells) were collected, washed with MACS buffer and resuspended in complete IMDM prior to use in sections 2.8, 2.10 and 2.11.

2.5 Isolation of Monocytes

Unlabelled monocytes were isolated from PBMCs by negative selection using monocyte isolation kit II (Miltenyi Biotec, UK) according to the manufacturer's instructions. The purity of $CD14^+CD16^-$ cells was >94%. In brief, PBMCs were resuspended in 30 µl MACS buffer (PBS Ca^{2+} and Mg^{2+} free, with 0.5% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.2, sterile filtered) per 10^7 cells, blocked with Fc receptor blocking Reagent for 5 minutes, and incubated with 10 µl of biotin-antibody cocktail per 10^7 cells for 10 minutes at 4^oC. A further 30 µl

of MACS buffer and 20 µl of anti-biotin MicroBead Cocktail were added per 10^7 cells and incubated for 15 minutes at 4°C. The cells were washed with 2 ml of MACS buffer (centrifuge at 300g for 10 mins), resuspended in 500 µl of MACS buffer per 10^8 cells and separated with LS columns (Miltenyi Biotec) placed in the magnetic field of a suitable MACS separator. The unlabelled cell flow through (enriched monocytes) were collected, washed with MACS buffer and resuspended in complete IMDM prior to use in sections 2.6 and 2.12.

2.6 Differentiation and Activation of Macrophages from Monocytes

2 x 10^5 purified $CD14^+$ monocytes were purified as above and plated on 6-well plates (GBO, UK) per well in completed IMDM, and incubated for 1 hour at 37°C in a humidified 5% CO_2 incubator. Monocytes adhered to the tissue culture plastic. Non-adherent cells were removed by gently washing the monocyte monolayer with warm (37°C) IMDM three times. GM-CSF (100 ng/ml, R&D systems, UK) was added to the cells and cultured for 6 days without medium change to allow macrophage differentiation. To activate the immature monocyte-derived macrophages, the medium was replaced and LPS (10 ng/ml, Sigma Aldrich) was added at day 7 and cells were cultured for a further 24 hours. The cells were harvested with Trypsin/EDTA (0.05%, Gibco®, Thermo Fisher Scientific) and washed (300g, 5min) with PBS, stained with appropriate antibodies and analysed by flow cytometry on a FACS Calibur (BD Biosciences, Oxford, UK) (section 2.16), or the cells were resuspended in complete IMDM and co-cultured with H69 SCLC cells (section 2.12).

2.7 Mixed Leukocyte Reactions (MLR)

Mixed leukocyte reactions (MLRs) were established from the PBMCs of two separate unrelated donors prepared as described above. SCLC cells (H69, H345 and H510) were washed with PBS by centrifugation at 300g for 5 minutes and resuspended in PBS at 1 x 10^6 cells/ml, and incubated with mitomycin C (Sigma-Aldrich, UK) at 50 µg/ml for 3 hours at 37^{o}C. It was confirmed that this produced the maximum suppression in SCLC cell proliferation with least loss in cell viability by Trypan blue solution (Sigma-Aldrich). The cells were washed three times in PBS (300g, 5min) prior to use and resuspended in complete IMDM. In a 96-well plate (GBO, UK), 5 x 10^4 PBMCs per donor per well were combined with Concanavalin (Con) A (Sigma-Aldrich, UK) at 5 µg/ml, and co-cultured with mitomycin-C-treated SCLC cells at a 1:1 ratio (i.e. 1 x 10^5 mixed leukocytes/well and 1 x 10^5 SCLC cells) in 200 µl complete IMDM at 37^{o}C in a humidified 5% CO_2 incubator for 72 hours. Cell proliferation was assessed by ^{3}H-thymidine incorporation (section 2.9.1).

2.8 Naïve/CD4$^+$ T cell activation

Purified naïve/CD4$^+$ T cells were enriched as described above. For activation experiments, in 96-well plates, 2 x 10^5 CD4$^+$ T cells per well were stimulated with immobilized anti-CD3 (0.125 µg/ml) (OKT3, pre-coated for 6 hours at 37^{o}C) and soluble anti-CD28 (1 µg/ml)(CD28.2) monoclonal antibodies (both from eBioscience, UK) in a volume of 200 µl per well. In 24-well plates (GBO, UK), 2 x 10^5 naïve/CD4$^+$ T cells per well were stimulated with pre-coated immobilized anti-CD3 (1.25 µg/ml) and soluble anti-CD28 (1 µg/ml) in 1 ml per well complete IMDM at 37°C in 5%CO_2 in a humidified incubator for 72 hours. For some

experiments, naïve/$CD4^+$ T cells were activated with Dynabeads CD3/CD28 T cell expander (One bead per cell, Gibco®, Thermo Fisher Scientific). In others, the cells were treated with phorbol myristate acetate (PMA) (100 ng/ml, Sigma-Aldrich) plus ionomycin (IONO) (50 ng/ml, Sigma-Aldrich). Cell proliferation was assessed by ^{3}H-thymidine incorporation (section 2.9.1).

2.9 Cell proliferation assays

2.9.1 ^{3}H-thymidine incorporation

Cells were cultured in triplicate for 48 hours and pulsed for an additional 24 hours with 37kBq [^{3}H]-thymidine (1 µCi, Perkin Elmer, Cambridge, UK) per well. Cells were then harvested with a Tomtec (Leamington Spa, UK) cell harvester and the radionuclide uptake was measured in a beta plate reader (Wallac, UK) by scintillation counting as an indicator of proliferation.

2.9.2 Carboxyfluorescein diacetate succinimidyl ester (CFSE) incorporation

To assess more details of $CD4^+$ T cell proliferation, freshly isolated naïve/$CD4^+$ T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit (1 µM; Molecular Probes™, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, isolated $CD4^+$ T cells were incubated with 1 µM CFSE in pre-warmed PBS/0.1%BSA for 10 minutes at 37^{o}C. The reaction was then quenched by addition of 5 volumes of ice-cold complete IMDM culture medium and incubated on ice for 5 minutes. The cells were immediately washed twice with complete IMDM, and then co-cultured with

SCLC cells using Transwells. After 72 hours, the cells were harvested and analysis of cell division was estimated by flow cytometry on a FACS Calibur (BD Biosciences, Oxford, UK) using FlowJo™ (Tree Star) analysis software.

2.10 Co-culture of SCLC cells and $CD4^+$ T cells

$CD4^+$ T cells were purified and SCLC cells were treated with mitomycin-C as described above. The cells were washed three times in PBS (300g, 5min) prior to use. In 96-well plates, 2 x 10^5 $CD4^+$ T cells were activated as described above. 2, 4 or 8 x 10^5 cells/well mitomycin-C treated H69, H345 or H510 were co-cultured with $CD4^+$ T cells for 72 hours at 37°C. After 48 hours 1 mCi ^{3}H-thymidine was added to each well. 24 hours later cells were harvested and cellular uptake of ^{3}H-thymidine was assessed by liquid scintillation counting using a beta plate reader (Wallac, Finland) (section 2.9.1).

2.11 Transwell co-culture of SCLC cells and naïve/$CD4^+$ T cells

For the co-culture experiments using transwells, polycarbonate 24-well transwell inserts (0.4 µM pore size) and receiver trays (both from Millipore, UK) were used to physically separate naïve/$CD4^+$ T cells and H69 SCLC cells. 2 x 10^5 naïve/$CD4^+$ T cells were resuspended in 800 µl complete IMDM, stimulated with immobilized anti-CD3 (1.25 µg/ml) and soluble anti-CD28 (1 µg/ml) and added in the lower chambers of the plates. 200 µl complete IMDM only or containing 2, 4 or 8 x 10^5 H69 SCLC cells (T cells/SCLC cell ratio of 1:1, 1:2 or 1:4) was added to the upper transwell chambers. The plates were incubated for 72 hours at 37°C. The cells were harvested for cell proliferation assay by uptake of ^{3}H-thymidine or CFSE incorporation (section 2.9), or for Treg cell population

analysis by flow cytometry (section 2.16). The cell culture supernatants were collected for cytokines measurement by ELISAs (section 2.15).

2.12 Transwell co-culture of SCLC cells and monocytes or MDMs

1 x 10^5 monocytes were purified from PBMCs or 1 x 10^5 monocyte-derived macrophages (MDMs) were differentiated as described above, resuspended in 800ul complete IMDM in 24-well plates. 200 µl complete IMDM alone or containing 4 x 10^5 H69 SCLC cells was added to the upper transwell chambers. After 24 hours incubation, H69 cells in transwells and cultured medium were removed, washed three times with PBS, and monocytes or MDMs were resuspended in 200 µl lysis buffer prepared as above, the cell lysates were collected for western blot (section 2.20).

1 x 10^5 purified monocytes were resuspended in 800 µl complete IMDM and activated with LPS (10 ng/ml) in the lower chambers of 24-well plates. 200 µl complete IMDM alone or containing 1, 2 or 4 x 10^5 H69 SCLC cells was added to the upper transwell chambers. In some experiments, RC-3095 (1 µM, Sigma-Aldrich) or Dimethyl sulfoxide (DMSO) (Sigm-Aldrich) was added to the culture. After 24 hours incubation, the supernatant was collected (300g, 5min) and TNF-α production was measured by ELISA (section 2.15).

Monocyte-derived macrophages (MDM) were differentiated as described above and harvested with Trypsin/EDTA (0.05%, Gibco®, Thermo Fisher Scientific) at day 6. 2 x 10^5 MDM were resuspended in 800 µl complete IMDM in the lower chambers of 24-well plates, stimulated with LPS (10 ng/ml), cultured alone or

co-cultured with 8 x 10^5 H69 SCLC cells in 200 µl IMDM in transwells in the presence of RC-3095 (1 µM, Sigma-Aldrich) or DMOS control (Sigm-Aldrich). Cells and supernatants were collected by centrifugation (300g, 5min) prior to analysis by flow cytometry (section 2.16) and ELISAs (section 2.15).

2.13 Culture of naïve/$CD4^+$ T cells with SCLC cell conditioned medium (CM)

5 x 10^5 purified naïve/$CD4^+$ T cells were resuspended in 600 µl complete IMDM and stimulated with immobilized anti-CD3 (1.25 µg/ml) and soluble anti-CD28 (1 µg/ml) in 24-well plate. H69 and H510 SCLC cell supernatants were prepared as described above and serum-free CM was removed. 400 µl of H69 CM, H510 CM or complete IMDM was added into each well containing $CD4^+$ T cells. In some experiments, H69 serum-free CM was heated to 56^{o}C for 10 minutes or was further separated into fractions containing soluble molecules < 30kDa or > 30kDa using centrifugal filter concentrators (Centricon®Plus-70, Millipore) according to the manufacturer's instructions. In brief, 50 ml H69 cell culture medium was added to the centrifugal filter devices, centrifuged at 3,500g for 10 minutes at room temperature. Each fraction was collected and added separately to the $CD4^+$ T cell cultures. Plates were incubated for 72 hours at 37^{o}C. The cells were harvested for cell proliferation assay by uptake of ^{3}H-thymidine or CFSE incorporation (section 2.9), or for Treg cell population analysis by flow cytometry (section 2.16). The cell culture supernatants were collected for cytokine measurement by ELISA (section 2.15).

2.14 Cell culture additives

Recombinant human IL-15 protein (rIL-15) (20 pg/ml, 20 ng/ml, 200 ng/ml, R&D Systems) was added as indicated to the activated naïve $CD4^+$ T cell culture alone or with H510CM. IL-10 signalling was blocked with monoclonal blocking anti-human IL-10 Rα antibody compared to isotype control antibody (mouse IgG_{2B}) at a concentration of 20 µg/ml (R&D systems). TGF-β signalling was blocked with monoclonal neutralising anti-TGF-β antibody (active against all isoforms) and compared to isotype control antibody (mouse IgG_1) at a concentration of 10 µg/ml (R&D systems) or with 1 µM of TGF-β and activin-signalling inhibitor SB431542 hydrate (Sigma-Aldrich) and DMSO vehicle control (Sigma-Aldrich). GRP signalling was blocked as indicated using a potent GRP receptor antagonist RC-3095 (1 µM, 5 µM, 10 µM, Sigma-Aldrich) or DMSO (Sigma-Aldrich) as control. IL-15 function was blocked with a monoclonal mouse anti-human IL15 neutralising antibody (200 ng/ml, eBioscience) compared to the isotype control mouse IgG1 antibody.

2.15 Measurement of cytokine production by Enzyme Linked Immunosorbent Assay (ELISA)

1 x 10^6 H69 SCLC cells were cultured in serum-free IMDM in 24-well plates for 72 hours, and then the supernatant was collected and centrifuged (300g, 5mins) prior to TGF-β1 ELISA.

For co-culture of $CD4^+$ T cells with SCLC cells in transwells, 2 x 10^5 cells $CD4^+$ T cells were stimulated with anti-CD3/CD28 in 24-well plates, co-cultured with 2, 4 or 8 x 10^5 live H69 SCLC cells in transwells. In some experiments, 5 x 10^5 $CD4^+$ T cells were stimulated with anti-CD3/CD28 in 24-well plates and cultured

with serum-free H69 CM diluted to 20% or 40%. The supernatants from $CD4^+$ T cells were collected and centrifuged (300g, 5min) after 72 hours stimulation, the cytokines IL-10, INF-γ, IL-4, and IL-17 were all measured in the supernatants of co-culture of $CD4^+$ T cells and SCLC cells, or the supernatants from $CD4^+$ T cells cultured with SCLC conditioned medium by ELISA (all from R&D systems, UK), IL-15 by Ready-SET-Go!® (eBioscience, UK) according to the manufacturer's instructions. For co-culture of monocytes or monocyte-derived macrophages (MDMs) with SCLC cells in transwells, 1 x 10^5 monocytes or 2 x 10^5 MDM were co-cultured alone or with 4 x 10^5 or 8 x 10^5 SCLC cells for 24 hours in the presence of LPS at a concentration of 10 ng/ml, the supernatants were collected (300g, 5min) and cytokines TNF-α, INF-γ, IL-6 and IL-10 were measured by ELISAs (R&D systems). The supernatants were either analysed immediately or stored frozen until analysis. Plates were read at 450 nm (650 nm reference) on a Biotek Synergy HT plate reader (Fisher Scientific, UK).

2.16 Flow cytometric analysis of protein expression

Surface marker staining used standard protocols with conjugated mouse monoclonal antibodies: FITC-anti-CD8 (DK25), RPE-anti-CD4 (MT310), RPE-Cy5-anti-CD3 (UCHT1) and mouse IgG1 (DAK-GO1) isotype controls (all obtained from DAKO); PE-anti-CD45RA (HI100) and PE-anti-CD127 (eBioBDR5) (both obtained from e-Bioscience). For regulatory T cell ($CD4^+CD25^+Foxp3^+$) staining, human Treg detection kit (Miltenyi Biotec) was used according to the manufacturer's instructions. Briefly, fresh isolated, or anti-CD3/CD28 activated alone, or anti-CD3/CD28 activated and co-cultured (with H69 cells or H69 CM) $CD4^+$ T cells were stained against surface antigens with

FITC-anti-CD4 (VIT4) and PE-anti-CD25 (4E3) conjugated monoclonal antibodies. The cells were then fixed and permeabilized, blocked with Fc receptor (FcR) blocking reagent and stained with intracellular APC-anti-FOXP3 (3G3) or mouse IgG1 isotype control. Alternatively, Tregs were stained with FITC-anti-CD4 (VIT4), PE-anti-CD127 (eBioBDR5) and APC-anti-FOXP3 (3G3). In some experiments, fresh isolated $CD4^+$ T cells were labelled with CFSE as above, co-cultured with H69 cells at the ratio of 1:4 in transwells for 72 hours, then fixed and permeabilized. The cells were then treated with FcR blocking reagent, stained with PE-anti-mouse/human Helios protein (22F6; Biolegend, UK) and APC-anti-human FOXP3 (3G3) or isotype control and analyzed by flow cytometry.

For monocyte-derived macrophage (MDM) surface marker staining, the cells were blocked with Fc receptor blocking solution (BioLegend, UK), and stained with FITC-anti-CD16 (CB16), Alexa-Fluor488 anti-CD86 (IT2.2), PE-anti-CD14 (61D3), PE-anti-CD163 (GHI/61), PE-Cyanine7-anti-CD68 (Y1/82A), PerCP-eFluor710-anti-CD80 (2D10.4), APC-anti-HLA-DR (LN3). APC-anti-CD206 (19.2) (all obtained from eBioscience) or mouse IgG1 isotype control antibody. The cells were either analyzed immediately or fixed with 2% Paraformaldehyde solution (Sigma-Aldrich) for 15 minuets at room temperature and analyzed within 24 hours by flow cytometry. Data were acquired on a FACS Calibur flow cytometer and analysed with FlowJo™ software.

2.17 Treg functional assay

Naïve $CD4^+$ T cells purified from single donors were divided into two aliquots. One aliquot was stimulated as above with anti-CD3/CD28 antibodies, then co-cultured with H69 SCLC cells at 1:4 ratio in transwells for 3 days. The second aliquot was immediately frozen and stored at -80^oC for 3 days in freezing medium: 40% complete IMDM, 50% FBS and 10% DMSO (Hybri-Max^{TM}, Sigma-Aldrich).

On day 3, frozen naïve $CD4^+$ T cells were quickly thawed (37^oC, 5 min) and washed three times with warm complete IMDM by centrifugation (300g, 5min). The cells were assessed by trypan blue assay (Sigma-Aldrich) to have > 95% viability. They were then labelled with CFSE. The previously activated, co-cultured autologous $CD4^+$ T cells were harvested and counted. 1 x 10^5 cells/well CFSE-labelled defrosted naïve $CD4^+$ T cells were then co-cultured with 2 x 10^5 cells/well of unlabelled autologous $CD4^+$ T cells in 24-well plates in the presence of Dynabeads^{TM} CD3/CD28 T cell expander (one bead per cell, Thermo Fisher Scientific). After 3 days, the proliferation and cell division of CFSE-labelled $CD4^+$ T cells was analysed by flow cytometry.

2.18 Immunohistochemistry (IHC)

Ethical and institutional management approval for the study was obtained from the Lothian Research Ethics committee. 64 biopsy samples with primary lung SCLC from the years 1999 to 2001, for which sufficient residual material and the clinical data were available, were identified from the pathology archives at the Royal Infirmary of Edinburgh.

Formalin-fixed, paraffin-embedded lung tumour sections (3 μm) were de-waxed in xylene for 10 minutes. Tissue was rehydrated through graded ethanol (100%, 90%, 70%, 50%, 5min each). Antigen retrieval was performed by heating rehydrated tissue in BORG Decloaker pH9.5 (Biocare Medical, Walnut Creek, CA) using a 1000W microwave oven for 10 minutes at maximum power. Sections were cooled in running tap water for 10 minutes and endogenous peroxidase was blocked in 3% hydrogen peroxide (v/v in distilled water, Sigma-Aldrich) for 15 minutes at room temperature. Slides were mounted in a Sequenza® (Shandon) slide rack, washed with PBS, blocked with general protein block (DAKO) for 30 minutes at room temperature and followed by avidin block (Vector Labs) for 15 minutes at room temperature. Appropriate primary antibodies or isotype control antibodies were diluted in antibody diluent (DAKO): polyclonal rabbit ant-human CD3 (1:200 dilution, clone IS503, DAKO), monoclonal mouse anti-human CD4 (1:100 dilution, clone 4B12, DAKO), monoclonal mouse anti-human CD68 (1:100 dilution, clone PG-M1, DAKO), monoclonal mouse anti-human CD20cy (1:300 dilution, clone L26, DAKO), monoclonal mouse anti-human CD45 (1:100 dilution, clone 2B11 + PD7/26, DAKO), monoclonal mouse anti-human FoxP3 (1: 25 dilution, clone 236A/E7, eBioscience), monoclonal mouse anti-human IL-15 (1: 500 dilution, Abcam), polyclonal rabbit anti-human GRP-R (1:200 dilution, Thermo Fisher Scientific). 100 μl of diluted primary antibody was added to each slide and incubated overnight at 4^{o}C. Slides were then washed with PBS and blocked with biotin block (Vector Labs) for 15 minutes at room temperature. The detection was performed with biotinylated secondary antibody (100 μl of Polyclonal goat anti-mouse, Polyclonal goat anti-rabbit, 1:400 dilution, DAKO), and incubated with a

preformed complex of avidin and biotinylated horseradish peroxidase macromolecular (Vector Labs) for 60 minutes at room temperature. The slides were washed with PBS, developed with 3,3V-diaminobenzidine DAB (100 μl, DAKO) according to the manufacturer's instructions for 5 minutes at room temperature in the dark. They were washed with PBS and counterstained with Mayers haematoxylin (Sigma-Aldrich) for 1 minute and acid for 5 seconds, immersed in Scotts tap water for 3 minutes. Dehydration was then achieved through graded ethanol (50%, 70%, 90%, 100%), cleared in xylene (5min each) and mounted with DPX mounting medium (Thermo Fisher Scientific). For IL-15 IHC staining on cytospins, H69 and H510 cells were washed with PBS and spun on slides (200 cells/mm3, 1000rpm, 5min). The cells were air-dried and fixed in 90% anhydrous acetone/10% methanol for 20 minutes at room temperature. The slides were washed and IL-15 immunostaining was performed as described above but without antigen retrieval. Stained slides were viewed with a Leica DM1000 microscope at 100-400X magnifications and photographed with a Leica DFC400 digital camera (both from Leica Microsystems, UK).

2.19 Immunofluorescence

SCLC tumour sections were de-waxed, rehydrated, and antigen retrieval was performed as described above. The sections were blocked with 5% goat serum for 30 minutes at room temperature，washed with PBS and incubated with polyclonal rabbit ant-human CD3 (1:200 dilution, clone IS503, DAKO) and monoclonal mouse anti-human Foxp3 antibody monoclonal mouse anti-human FoxP3 (1:25 dilution, clone 236A/E7, eBioscience). 100 μl of each primary antibody diluted in antibody diluent (DAKO) was added to the slide and

incubated for 1 hour at room temperature in the dark. The slides were washed with PBS and incubated with 100 μl of secondary antibodies Alexa-488 goat anti-rabbit IgG and Alexa-568 goat anti-mouse IgG in antibody diluent (1:1000 dilution, Thermo Fisher Scientific) for 30 minutes at room temperature in the dark, and followed by 100 μl of 4,6-diamidino-2-phenylindole (DAPI) (0.1 μg/ml, Thermo Fisher Scientific) to stain nuclei for 5 seconds and washed with PBS. The sections were mounted in aqueous mounting medium (DAKO) and analysed on a Leica TCS SP5 confocal microscope.

2.20 Western Blot

The cells were washed twice with PBS (300g, 5min), resuspended in 1X lysis buffer (Cell Signalling Technology) with 1mM PMSF (Sigma-Aldrich), 1X MINI protease inhibitor cocktail tablet (Roche), and agitated on ice for 30 minutes. Protein concentration in the whole cell lysates was measured using BCA protein assay reagent (Pierce™, Thermo Fisher Scientific) according to the manufacturer's instructions. Balanced whole cell lysates were resuspended in 4X premixed Laemmli protein sample buffer (277.8 mM Tris-HCl, pH 6.8, 4.4% LDS, 44.4% (w/v) glycerol, 0.02% bromophenol blue, Bio-Rad, UK) and denatured by heating to 95°C for 5 minutes. Lysates (15 μg protein per lane) were resolved on 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (NuPAGE™, Thermo Fisher Scientific) (120V, 40mA, 90min) and transferred onto 0.45 mm nitrocellulose membranes (Bio-Rad) (100V, 390mA, 60min). Blots were blocked with blocking buffer (5% w/v milk in 1X TBS, 0.1% Tween20) for 60 minutes at room temperature and

incubated with appropriate primary antibodies: polyclonal rabbit anti-human GRP-R (1:1000 dilution, Thermo Fisher Scientific), rabbit anti-human phospho-Akt (Ser473) (1:1000 dilution, Cell Signalling Technology), rabbit anti-human Akt (1:1000 dilution, Cell Signalling Technology) and rabbit anti-human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3000 dilution, Cell Signalling Technology) diluted in blocking buffer overnight at 4^{o}C. The membranes were incubated with horseradish peroxidase-labelled polyclonal goat anti-rabbit secondary antibody (1:3000 dilution, DAKO) diluted in blocking buffer for 60min at room temperature. The blots were detected using ECL western blotting detection reagent (Amersham) according the manufacturers instructions and visualised on the Bio-Rad ChemiDoc^{TM} MP System (Bio-Rad).

2.21 Cell viability determination by MTT assay

A (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) cell proliferation assay kit (Vybrant®, Thermo Fisher Scientific) was used to determine cell viability according to the manufacturer's instructions. The 24-well culture plates containing cells were centrifuged (300g, 5min) at room temperature; the culture medium was carefully removed and replaced with 500 μl complete medium. 50 μl of 5 mg/ml MTT stock solution was added to each well and incubated for 2 hours at 37°C. The medium containing MTT was discarded by centrifugation (300g, 5min) and the plates were left to dry for 30 minutes at room temperature. 500 μl of DMSO (Sigma-Aldrich) was added to each well, mixed thoroughly and incubated for 10 minutes at 37°C. 50μl of each sample was transferred in 96-well plates and absorbance at 560 nm was measured on a Biotek Synergy HT plate reader (Fisher Scientific, UK).

2.22 Microarray analysis

The comparative analysis was conducted by the bioinformatics team in the BHF Centre for Research Excellence, University of Edinburgh. Expression data were downloaded from the Gene Expression Omnibus. All result sets were derived from variants of the Affymetrix Human GenomeU133 GeneChip. Expression values were converted to the linear scale where appropriate. Annotation for the probe identifiers on these chips was derived from appropriate annotation packages provided by the Bioconductor software suite (www.bioconductor.org). All data were uploaded to a relational database and examined by use of a web-based query builder. Although the vast majority of probe identifiers were comparable between chips, this web interface allowed qualitative comparison of expression for different probe IDs mapping to the same gene via their gene IDs from Entrez (PMID 15608257). Because these data were derived from disparate experiments and not cross-normalised, this methodology allows only for a coarse comparison of expression levels.

2.23 Statistical analysis

Data were expressed as the standard error of the mean (± SEM). The statistical significance of the difference between two groups was performed using the Student's *t* test. For multiple group comparisons, one-way analysis of variance (ANOVA) with Tukey's post test was performed. Correlation between values was evaluated using nonparametric Spearman's rank correlation. All the statistical analysis was determined using GraphPad™ Prism 6 (Graph Software, San Diego, CA, USA) and probability values (*p*) of less than 0.05 were considered statistically significant.

Chapter 3: Small Cell Lung Cancer Cells Inhibit $CD4^+$ T Cell Proliferation

Chapter 3 Small Cell Lung Cancer Cells Inhibit $CD4^+$ T Cell Proliferation

3.1 Introduction

The immune system protects the host from tumour development, growth and metastasis, and has become an attractive target for the development of novel immunotherapy for cancer (178). To this end clinical trials of anti-tumour vaccines have been conducted. These have demonstrated that anti-tumour immunity can be induced or enhanced with beneficial effects in multiple cancers, however success rates remain limited (179,180). This may relate to insufficient presentation of appropriate “danger signals” needed to activate the immune system by tumour cells (181). Relatedly, tumour cells may remain so similar to the normal cells from which they were derived that they resist full recognition by the immune system (182). Once an immune response has been induced however, tumour cells may still escape from effective immunosurveillance, suggesting tumour-induced alterations to the patient’s immune system may subvert or suppress anti-tumour responses within tumour microenvironment and thus facilitating tumour growth (178,179).

SCLC may represent a good example of such disease behavior. In SCLC, most patients are immunocompromised with poorly understood local and systemic immune defects that correlate with worse morbidity and mortality. Indeed, delayed hypersensitivity skin test reactivity and a decreased CD4/CD8 ratio in peripheral blood lymphocytes are associated with a worse prognosis in SCLC (160,161). Similarly, the peripheral blood lymphocytes from SCLC patients demonstrate significantly reduced proliferative responses to phytohemagglutinin and impaired secretion of IL-2 and macrophage-activating factor (162). In addition, proliferating lymphocytes isolated from tissue fragments from lung

biopsies of SCLC patients and cultured in IL-2 are ineffective in lysing autologous tumour cells. Conversely, lymphocytes from NSCLC patients are effective (165). Interestingly, the serum extracted from SCLC patients has been demonstrated to inhibit antibody synthesis and expression of T-cell membrane markers (172), and the supernatants from SCLC cell lines can suppress PBL proliferation in response to phytohemagglutinin or concanavalin A (173). Taken together these data support the hypothesis that SCLC cells may specifically suppress cell-mediated immune responses.

Therefore, further understanding of the mechanism by which SCLC cells suppress the host immune response is likely required to aid the development of truly effective immunotherapy for SCLC. $CD4^+$ T cells have been shown to play a central role in anti-tumour immune responses (174). *In vivo* vaccination tumour challenge experiments demonstrate that immunization of $CD4^{-/-}$ knockout mice fail to prime a systemic immune response capable of rejecting this tumor challenge. In contrast, a significant fraction of $CD8^{-/-}$ knockout mice mount successful tumour rejection responses (174). These data suggest that anti-tumour immunity requires coordination by $CD4^+$-dependent effector cells. I first focused on the effects of SCLC cell lines upon PBMCs and $CD4^+$ T cells in co-culture.

3.2 Results

3.2.1 SCLC cells suppress proliferation of a Mixed Leukocyte Reaction.

The mixed leukocyte reaction (MLR) is driven by recognition of disparate MHC-II antigens on antigen-presenting cells, by $CD4^+$ T cells. It is a widely used *in vitro* assay for functional studies of cellular immunity (183). It was therefore used to determine whether the local interaction of SCLC cells with immune cells could suppress cell-mediated immune responses. MLRs were established by combining equal numbers of PBMCs from two separate donors treated with Con A to stimulate the cells. The MLRs were then co-cultured with H69, H345 or H510 SCLC cells, treated with mitomycin C to stop their proliferation. Proliferation of responder cells within each MLR was determined at 72 hours by measuring uptake of ^{3}H-thymidine.

As expected, MLRs stimulated with Con A (5 mg/ml) actively incorporated ^{3}H-thymidine at 72 hours, whilst SCLC cell lines (H69, H345 and H510) did not (Fig. 3.1). Interestingly, co-culture of mitomycin C-treated H69 and H345 SCLC cells significantly reduced ^{3}H-thymidine uptake in MLR, whilst H510 cells did not (Fig. 3.1). These data therefore suggest that MLR suppression is not due to non-specific effects of co-culture such as use of nutrients by SCLC cells or toxic suppression by mitomycin C. Instead they indicate that some SCLC cell lines differentially suppress cell-mediated immune responses via specific mechanisms.

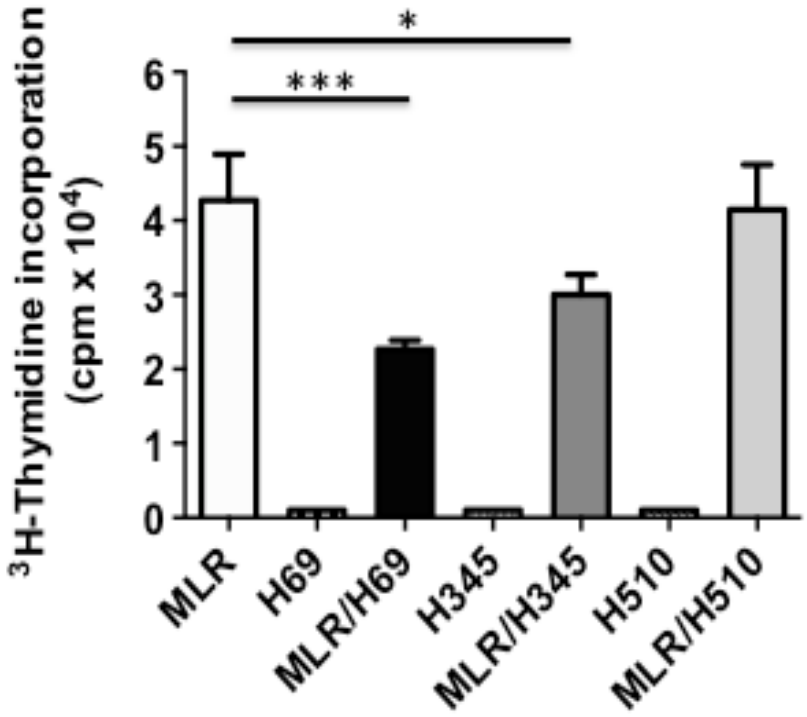
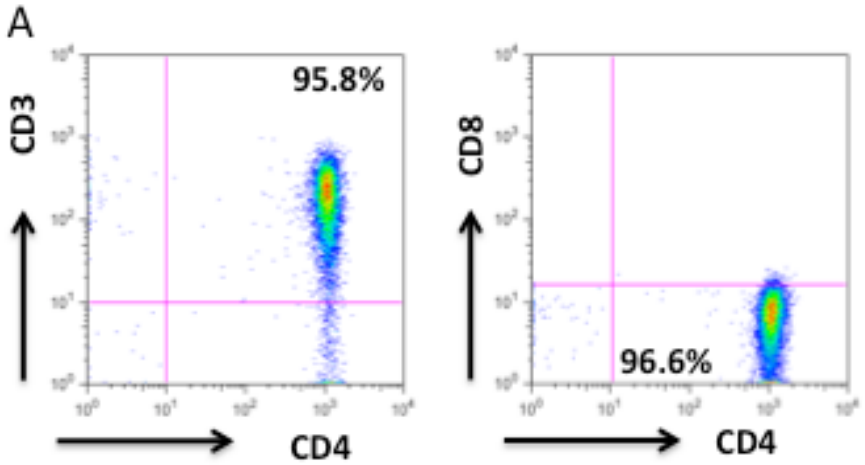


Figure 3.1: SCLC cells suppress proliferation of a Mixed Leukocyte Reaction. The experiments were performed as described in section 2.7 Chapter 2. Cell proliferation was assessed by ^{3}H-thymidine uptake (counts per minute; cpm) by the responder cells (mean ± SEM, n=7 experiments). * Indicates p <0.05 and *** p <0.0001.

3.2.2 H69 SCLC cells suppress proliferation of CD4$^+$ T cells.

The mixed leukocyte reaction involves many different cell types. For anti-tumour immune responses CD4$^+$ T cells are believed to be critical (174,184). Consequently suppression of CD4$^+$ proliferation and/or modulation of their immune behaviour may be an effective mechanism by which tumour cells can evade such responses. Therefore, to test this hypothesis in the context of the differential immunomodulatory effects of SCLC co-cultures with MLRs, the effects of different SCLC cell lines on CD4$^+$ T cells were next studied.

To this end, a novel *in vitro* co-culture system of SCLC cell lines and $CD4^+$ T cells was established. $CD4^+$ T cells were first isolated and purified to > 95% homogeneity (Fig. 3.2A). Proliferation of the $CD4^+$ T cells following activation with anti-CD3/CD28 and of H69, H345 and H510 SCLC cell lines treated with mitomycin-C either alone or in combinations of stimulated $CD4^+$ T cells was assessed. As expected, $CD4^+$ T cells activated with anti-CD3/CD28 produced a marked uptake of ^{3}H-thymidine, whilst the SCLC cell lines treated with mitomycin-C had no ^{3}H-thymidine uptake. The co-culture studies followed the same pattern of differential immunomodulation between SCLC lines treated with mitomycin-C as was observed for MLRs with PBMCs. Co-culture of activated $CD4^+$ T cells with H69 cells significantly reduced ^{3}H-thymidine uptake (Fig. 3.2B and 3.2C). Although co-culture with H345 cells also reduced $CD4^+$ T cells ^{3}H-thymidine uptake by $CD4^+$ T cells at day 3, the difference between the means was not significant (Fig. 3.2B). H510 SCLC cells had no observable effect on $CD4^+$ T cell proliferation in response to anti-CD3/CD28 (Fig. 3.2B). The data demonstrate that H69 SCLC cells can inhibit $CD4^+$ T cell proliferation whilst H510 cells cannot.



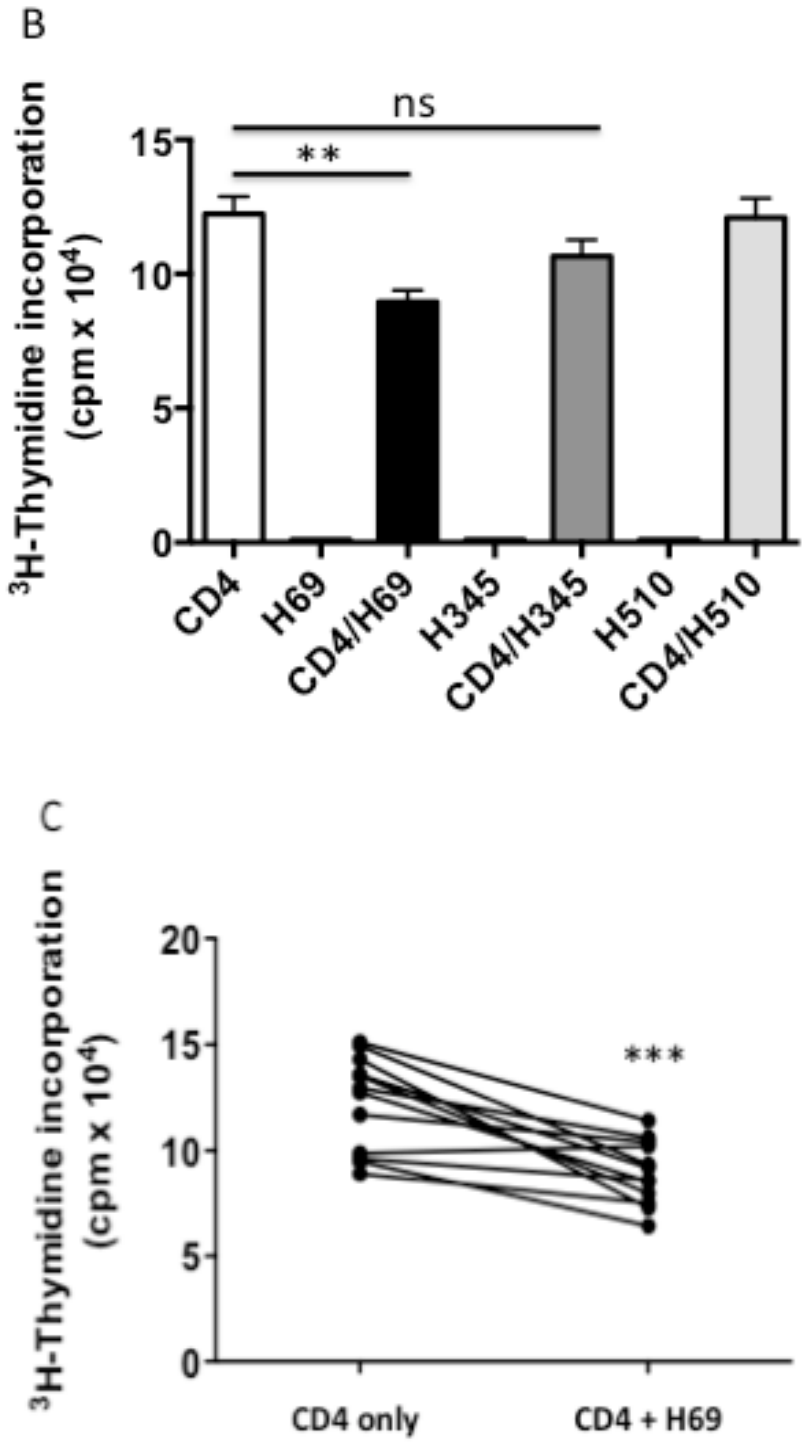


Figure 3.2: H69 SCLC cells suppress proliferation of $CD4^+$ T cells. The experiments were performed as described in section 2.10 Chapter 2. The data shows the proliferation of $CD4^+$ T cells alone or in co-culture with SCLC cell lines. (A) Representative flow cytometry shows the populations of purified $CD3^+CD4^+$ T cells and $CD4^+CD8^-$ T cells. (B) Mean ^{3}H-thymidine uptake (counts per minute; cpm) ± SEM by SCLC cells and activated $CD4^+$ T cells culture alone or with SCLC cell lines (H69 n=12, H345 n=9, H510 n=6). ** Indicates p <0.01 (t-test). (C) Uptake of

^{3}H-thymidine by activated CD4^{+} T cells cultured alone or with equal number of H69 cells (mean ± SEM, n=12 experiments). *** Indicates p <0.0001 (one-way ANOVA).

3.2.3 H69 SCLC cells suppress CD4^{+} T cell proliferation in a dose-dependent fashion.

In the tumour microenvironment, the ratio of SCLC cells to CD4^{+} T cells is frequently greater than 1:1 (Fig. 6.1). To investigate whether SCLC cells suppress the CD4^{+} T cell proliferation in a dose-dependent fashion, CD4^{+} T cells were co-cultured with increased ratios (CD4:H69 ratio: 1:1; 1:2; 1:4) of mitomycin C treated H69 SCLC cells. As expected, CD4^{+} T cells stimulated with anti-CD3/CD28 alone produced active uptake of ^{3}H-thymidine, and co-culture with H69 SCLC cells at the ratio of 1:1 resulted in a non-significant reduction in CD4^{+} T cell proliferation (Fig. 3.3A). However, importantly, co-culture with increased ratios (CD4:H69 ratio: 1:2 and 1:4) of H69 SCLC cells significantly suppressed CD4^{+} T cell ^{3}H-thymidine uptake compared to CD4^{+} T cells alone (Fig. 3.3A).

1:1 co-culture of CD4^{+} T cell with equal numbers of H345 and H510 did not show significant effects in reducing proliferation (Fig. 3.2B). However, given the dose-dependent of such effects with H69 cell co-culture, it was therefore important to address whether greater numbers of these SCLC cell lines could suppress CD4^{+} T cell proliferation. Co-culture of CD4^{+} T cells in a 1:4 ratio with H345 SCLC cells significantly reduced CD4^{+} T cell ^{3}H-thymidine uptake compared to CD4^{+} T cells alone (Fig. 3.3B). This was consistent with the non-

significant trend to suppression of $CD4^+$ T cell proliferation observed with 1:1 co-culture with H345 cells (Fig. 3.2B). Conversely, 1:4 co-culture with H510 cells continued to show no indication of a suppressive effect upon $CD4^+$ T cell proliferation (Fig. 3.3B).

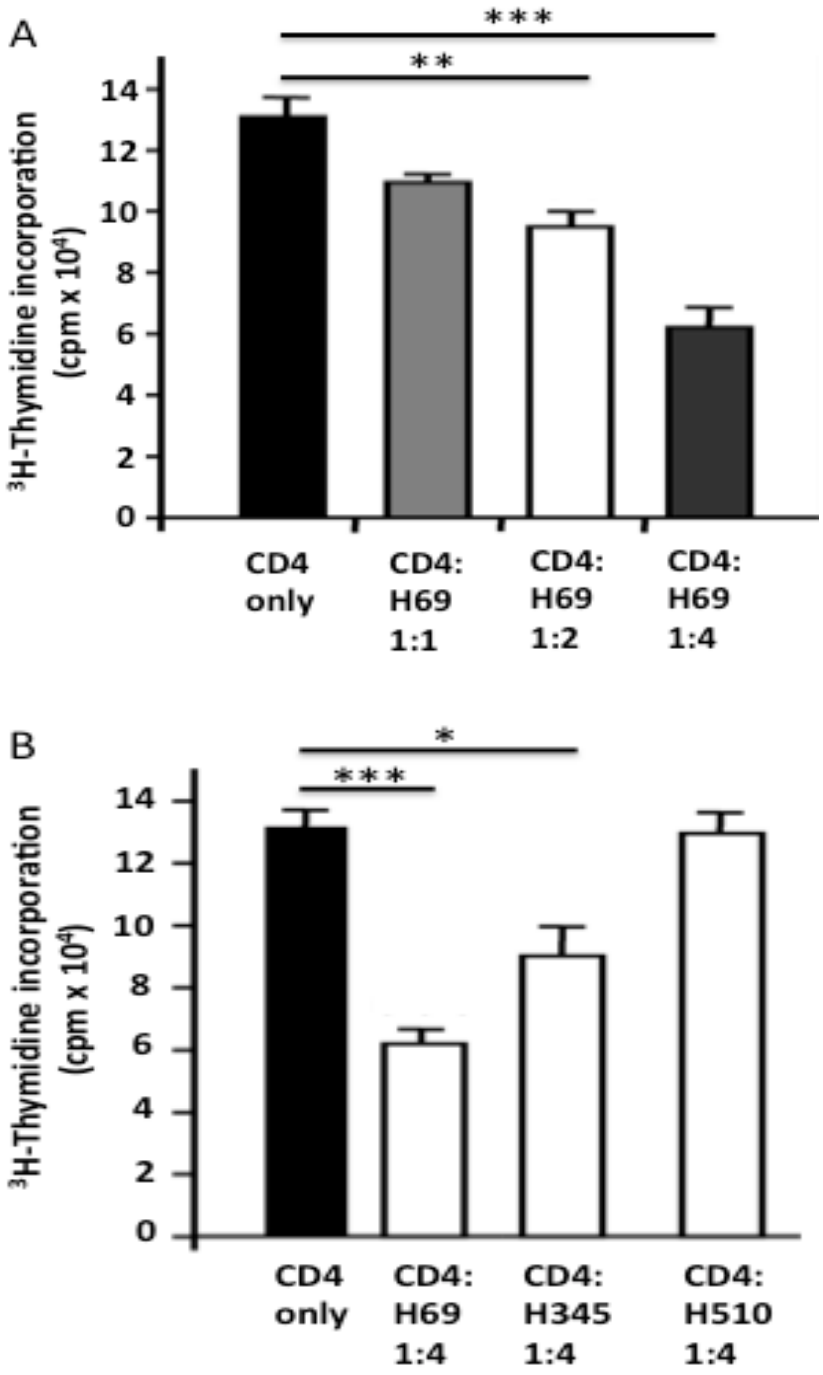


Figure 3.3: H69 SCLC cells suppress $CD4^+$ T cell proliferation in a dose-dependent fashion. The experiments were performed as described in section 2.10 Chapter 2. (A) Uptake of ^{3}H-thymidine

(counts per minute; cpm) by $CD4^+$ T cells cultured alone or with H69 SCLC cells (CD4: H69 1:1. 1:2, 1:4) (mean ± SEM, n=6 experiments). ** Indicates p <0.01 and *** p <0.0001. (B) Uptake of ^{3}H-thymidine (counts per minute; cpm) by $CD4^+$ T cells cultured alone or with H69, H345 or H510 SCLC cells (mean ± SEM, n=6 H69, n=4 H345 and n=4 H510). * Indicates p <0.05 and *** p <0.0001.

3.2.4 Transwell co-culture of H69 SCLC cells suppresses $CD4^+$ T cell proliferation.

I next investigated whether H69 SCLC cells suppressed $CD4^+$ T cell proliferation through contact-dependent inhibition or by the effects of secreted paracrine factors. This was assessed by modified co-culture experiments using transwell inserts that prevent direct contact between $CD4^+$ T cells and live H69 SCLC cells in the culture. $CD4^+$ T cells were stimulated with anti-CD3/CD28, cultured alone or co-cultured with live H69 cells at T cell to H69 cell ratios 1:1, 1:2 and 1:4. After 72 hours, the transwell inserts and their SCLC cell contents were removed and $CD4^+$ T cell proliferation was measured by uptake of ^{3}H-thymidine. Co-culture with increased ratios of H69 cells in transwells significantly reduced the proliferation of activated $CD4^+$ T cells at a 1:1 ratio and this effect was increased in a dose dependent manner (Fig. 3.4). These effects were highly similar to those observed with unsegregated co-culture (Fig. 3.3). The data therefore indicate that H69 SCLC cells inhibit $CD4^+$ T cell proliferation predominantly due to the paracrine effects of soluble factors produced by H69 cells rather than in a contact-dependent manner.

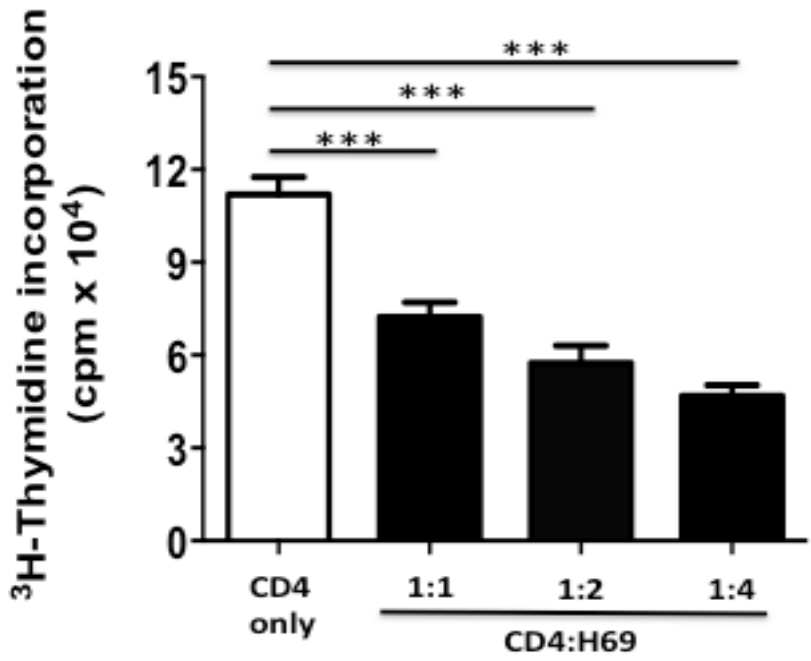
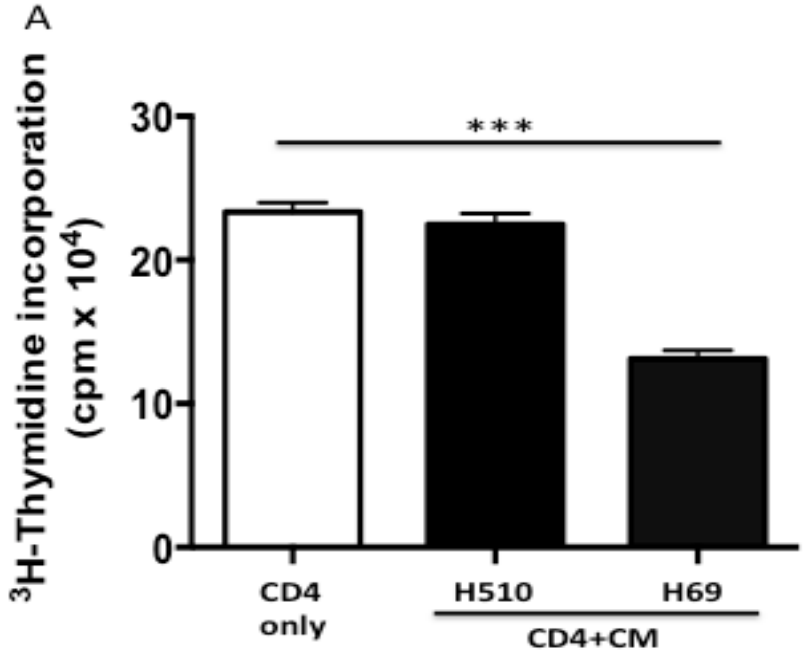


Figure 3.4: Transwell co-culture of H69 SCLC cells suppresses $CD4^+$ T cell proliferation. The experiments were performed as described in section 2.11 Chapter 2. Uptake of ^{3}H-thymidine (counts per minute; cpm) by $CD4^+$ T cells (mean ± SEM, n=9 experiments). *** Indicates p <0.0001. The raw data values are listed in table 3.4.

3.2.5 H69 SCLC conditioned medium (CM) suppresses $CD4^+$ T cell proliferation.

I next wished to determine whether the soluble factors mediating the suppression of $CD4^+$ T cell proliferation were constitutively produced by H69 cells. Serum-free conditioned medium from H69 or H510 cell culture was therefore added to activated $CD4^+$ T cell culture. Replacement of 40% of the culture medium with H510 cell culture supernatant did not affect $CD4^+$ T cell proliferation whilst replacement with H69 supernatant did (Fig. 3.5A). These data indicate that H69 SCLC cells continuously produce soluble factors that are able to suppress $CD4^+$ T cell proliferation. These effects were dose-dependent

with a significant ($p < 0.05$) difference between the degrees of suppression observed when 20% and 40% of the media were replaced (Fig. 3.5B). The consistent lack of any observable effect with H510 co-cultures or supernatant is most simply rationalised by the hypothesis that the soluble factors secreted by H69 SCLC cells are not sufficiently secreted by H510 cells. By the same logic, H345 cells might be predicted to secrete such factors at levels intermediate between H69 and H510 cells.



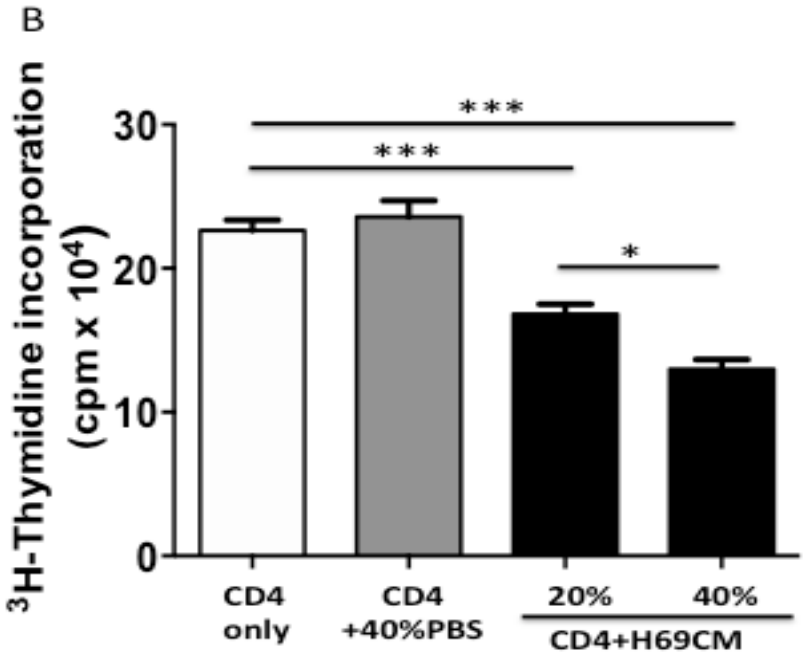


Figure 3.5: H69 SCLC cell conditioned medium (CM) supresses $CD4^+$ T cell proliferation. The experiments were performed as described in section 2.13 Chapter 2. (A) Uptake of ^{3}H-thymidine (counts per minute; cpm) by $CD4^+$ T cells cultured alone or with 40% of H510CM or H69CM (mean ± SEM, n=7 experiments). *** Indicates p <0.0001. (B) Uptake of ^{3}H-thymidine (counts per minute; cpm) by $CD4^+$ T cells cultured alone or with 40% of PBS, 20% or 40% H69CM (mean ± SEM, n=7 experiments). * Indicates p <0.05 and *** p <0.0001.

3.2.6 The suppression of $CD4^+$ T cell proliferation is regardless of the mechanism of stimulation.

Proliferation of $CD4^+$ T cells depends upon stimulation of both T cell receptor (TcR) and co-stimulatory signals. To assess whether H69 SCLC cell mediated inhibition of this process only occurs in the context of TcR stimulation (i.e. treatment with anti-CD3/CD28). This was also assessed when $CD4^+$ T cells were stimulated with PMA (an activator of protein kinase C (PKC)) plus IONO (a

Ca^{2+} ionophore and mitogenic for PBMC). Such treatment (PMA/IONO) has been show to trigger human T cell activation and proliferation (185). With PMA plus IONO stimulation, H69CM showed the same suppressive effect on $CD4^+$ T cell proliferation compared with anti-CD3/CD28 stimulation (Fig. 3.6). The data indicate that inhibition of $CD4^+$ T cell proliferation by factors secreted by H69 cells occurs regardless of the mechanism of stimulation.

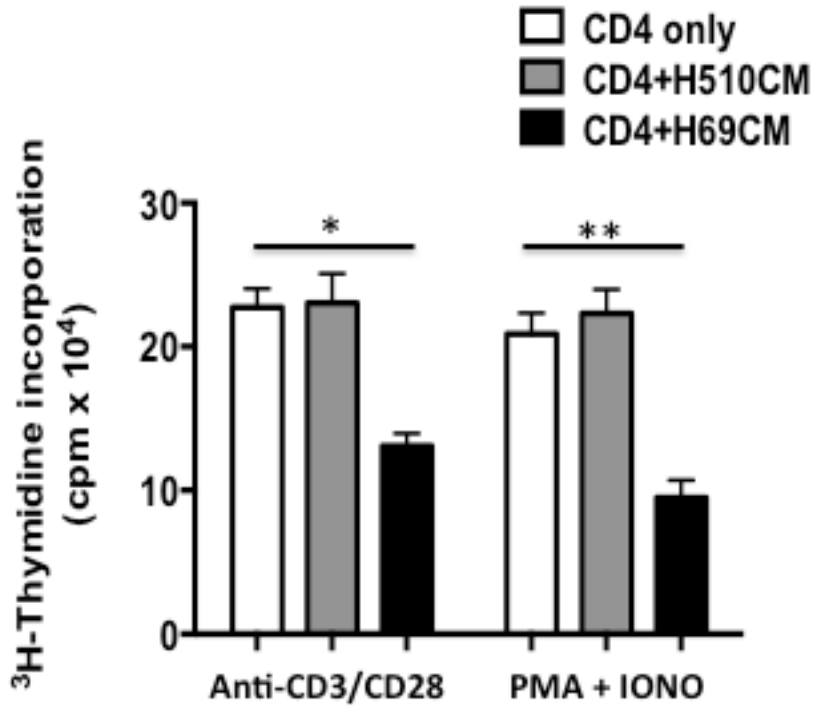


Figure 3.6: H69CM induced-suppression of $CD4^+$ T cell proliferation occurs in diverse stimulatory contexts. The cell proliferation was assessed by the uptake of ^{3}H-thymidine (counts per minute; cpm) by $CD4^+$ T cells with different stimulations in the presence of 40% H510CM or H69CM (mean ± SEM, n=3 experiments). * Indicates p <0.05 and ** p <0.01.

3.2.7 H69 SCLC cells produced soluble molecules that suppress $CD4^+$ T cell proliferation are less than 30kDa and heat stable.

To further investigate the nature of the soluble molecules, the ability of H69 supernatant to suppress $CD4^+$ T cell proliferation following either fraction on the basis of solute size, or heating, was assessed. The supernatant was filtered into fractions containing and excluding solutes > 30kDa. Fascinatingly the filtrate (i.e. enriched for solutes < 30kDa) retained the same ability to suppress proliferation as the unfractionated supernatant, whilst the remaining fraction did not (Fig. 3.7). These data indicate that the soluble factor(s) secreted by H69 cells is/are < 30kDa in size. Some cytokines have been shown to be resistant to inactivation by heat (186). Suppressive activity was similarly preserved following heating (56^{o}C for 10 minutes), indicating the property was due to one or more relatively thermostable factors. Together these findings suggest that small, heat stable cytokines may be candidate factors mediating the suppressive effect of H69 SCLC cells upon $CD4^+$ T cell proliferation.

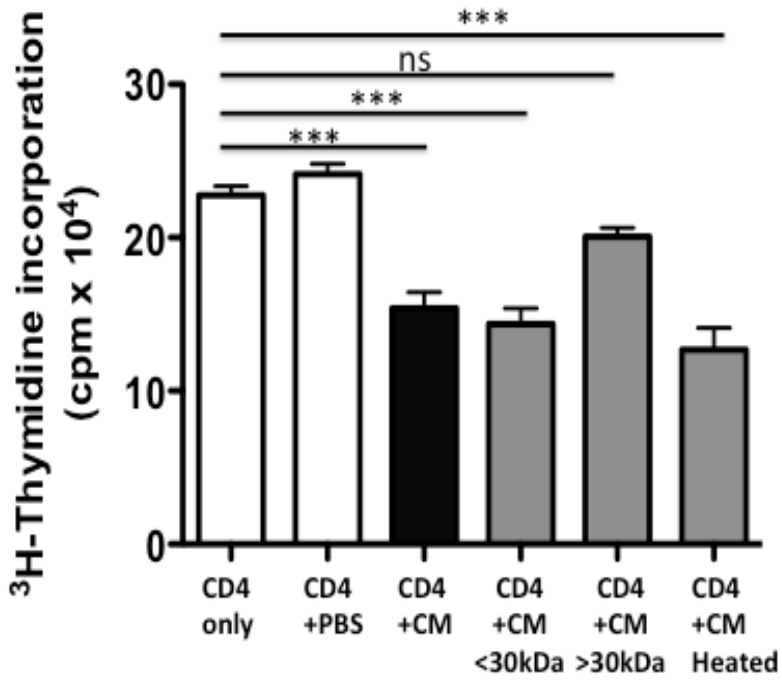


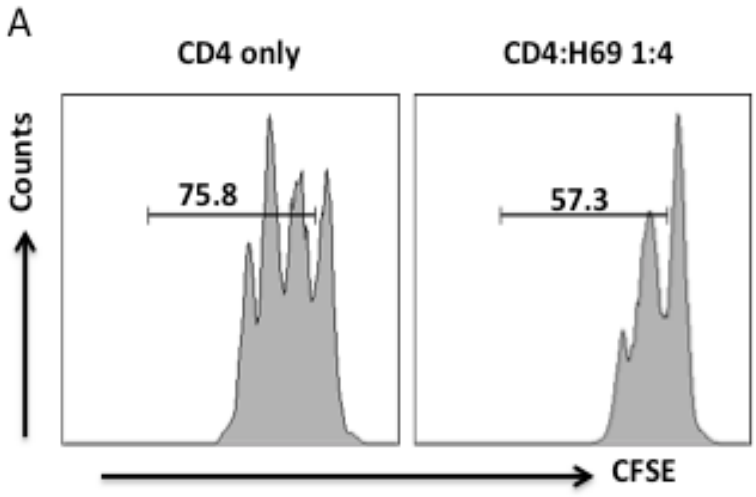
Figure 3.7: H69 SCLC cells produced soluble molecules that suppress $CD4^+$ T cell proliferation are <30kDa and heat stable. The experiments were performed as described in section 2.13 Chapter 2. Bar graph shows uptake of ^{3}H-thymidine (counts per minute; cpm) by $CD4^+$ T cells cultured with different fractions of 40% H69CM (mean ± SEM, n=3 experiments). *** Indicates *p* <0.0001.

3.2.8 H69 SCLC cells inhibit $CD4^+$ T cell proliferation by preventing activated $CD4^+$ T cells from entering the cell cycle.

To determine the percentage of dividing cells and numbers of cell divisions of the proliferation block mediated by the soluble molecule secreted by H69 SCLC cells, CFSE staining was performed. $CD4^+$ T cells were labelled with CFSE cell tracking dye immediately after purification, stimulated with anti-CD3/CD28 and co-cultured with SCLC cells in transwells at a ratio of 1:4 for 72 hours at 37^{o}C.

When cells divide, half of the fluorescent CFSE is transferred to daughter cells, allowing estimation of divisions by flow cytometry.

Consistent with the preceding data on $CD4^+$ T cell proliferation, the total percentage of activated $CD4^+$ T cells entering divisions was significantly reduced when co-cultured with H69 cells compared to monoculture (Fig. 3.8A and 3.8B). The proportions of activated $CD4^+$ T cells undergoing only a single division was higher in co-culture with H69 cells compared to the control. Conversely, the fractions of cells going on to two and three divisions were significantly reduced (Fig. 3.8C). The data suggest that the soluble factors responsible for suppression of $CD4^+$ T cell proliferation act by cell cycle blockade.



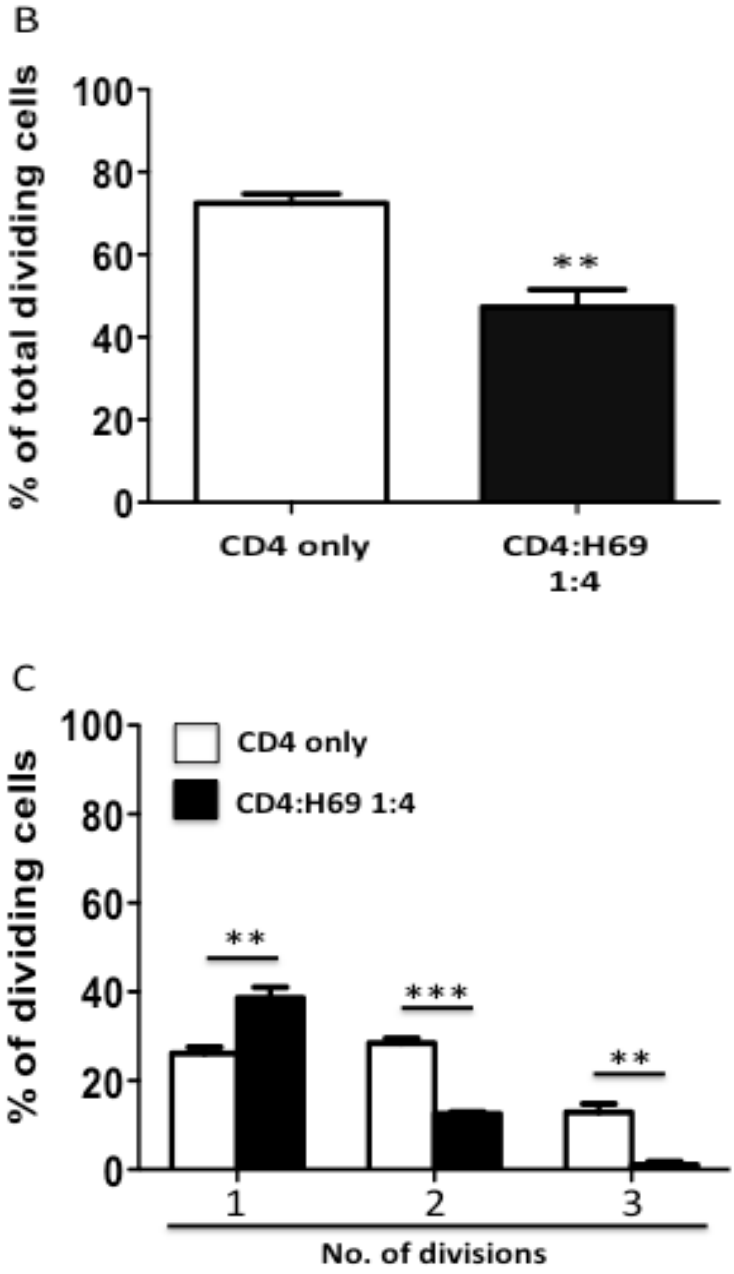


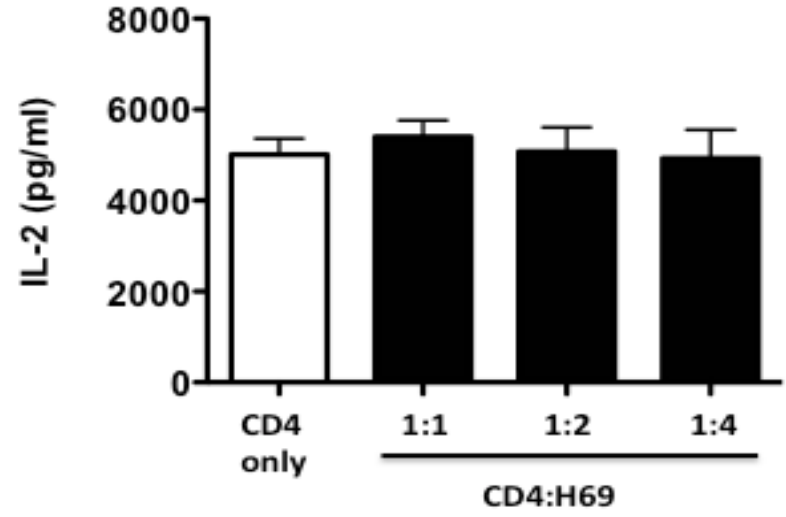
Figure 3.8: H69 SCLC cells inhibit $CD4^+$ T cell proliferation through preventing activated $CD4^+$ T cells from entering the cell cycle. The experiments were performed as described in section 2.9.2 Chapter 2. (A) Representative flow cytometry demonstrates the total percentage of dividing $CD4^+$ T cells. (B) The percentage of dividing $CD4^+$ T cells (mean ± SEM, n=4 experiments). ** Indicates p <0.01. (C) The percentage of $CD4^+$ T cells undergoing a single, two and three division cycles (mean ± SEM, n=4 experiments). ** Indicates p <0.01 and *** p <0.0001.

3.2.9 H69 SCLC cells suppress IL-17 but not IL-2, IFN-γ or IL-4 production.

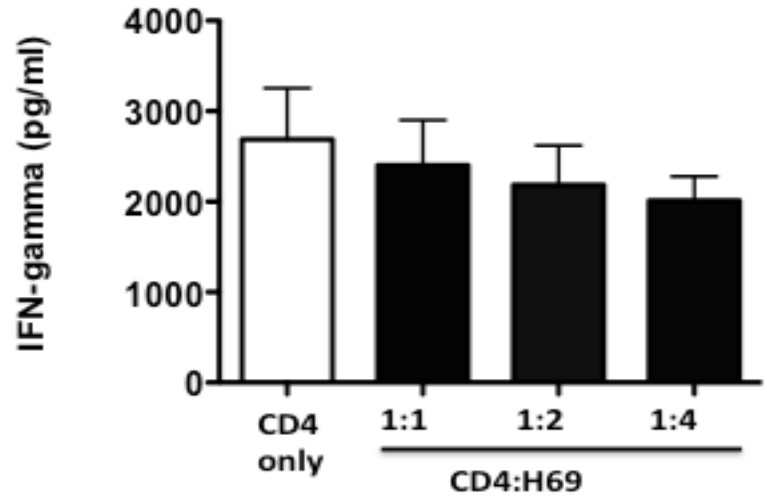
IL-2 is a potent T cell growth factor and can induce T cell expansion *in vitro* (187,188). IFN-γ and IL-4 are critical cytokines initiating the downstream signalling cascade to differentiate into Th1 and Th2 $CD4^+$ lineage cells respectively (189). IL-17 is produced by Th17 cells, which are a separate $CD4^+$ effector T helper cell subset (190). To determine whether the suppression of activated $CD4^+$ T cell proliferation was accompanied by changes in production of these cytokines, the levels at which they were released into cell culture supernatants were therefore examined.

Co-culture of activated $CD4^+$ T cells with H69 SCLC cells at 1:1 – 1:4 ratios did not significantly affect the levels of IL-2 (Fig. 3.9A), IFN-γ (Fig. 3.9B), or IL-4 (Fig. 3.9C) secreted into the supernatant. Interestingly, co-culture of activated $CD4^+$ T cells in 1:2 or 1:4 ratios with H69 cells resulted in significantly reduced IL-17 secretion (Fig. 3.9D). These data suggest that H69 SCLC cells do not readily induce a shift to a Th1 or Th2 phenotype in activated $CD4^+$ T cells *in vitro,* but may suppress a Th17 response.

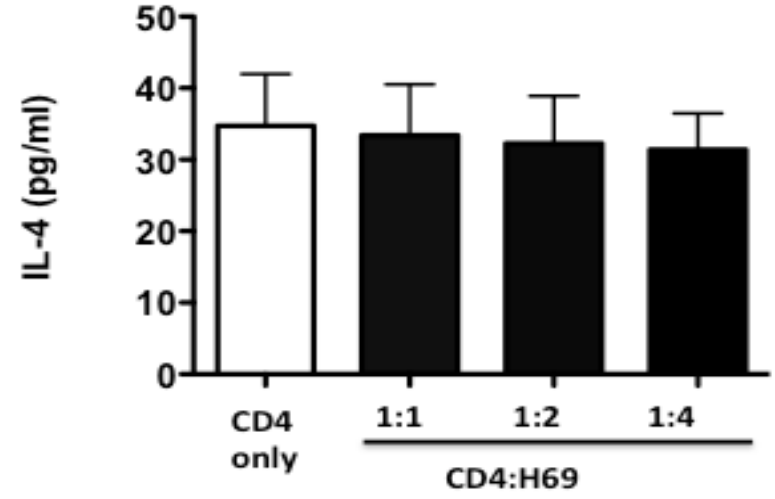
A


8000
6000
4000
2000
0
IL-2 (pg/ml)
CD4 only
1:1
1:2
1:4
CD4:H69

B


4000
3000
2000
1000
0
IFN-gamma (pg/ml)
CD4 only
1:1
1:2
1:4
CD4:H69

C


50
40
30
20
10
0
IL-4 (pg/ml)
CD4 only
1:1
1:2
1:4
CD4:H69

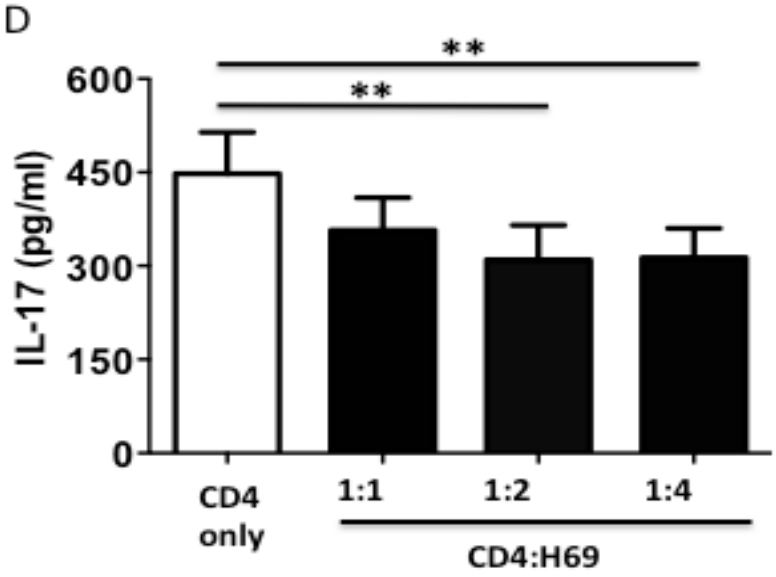


Figure 3.9: H69 SCLC cells suppress IL-17 but not IL-2, IFN-γ or IL-4 production. Cytokine production was measured as described in section 2.15 Chapter 2. Bar graphs show the concentration of IL-2 (A), INF-γ (B), IL-4 (C) and IL-17 (D) released by $CD4^+$ T cells in the supernatants (mean ± SEM, n=6 experiments). ** Indicates $p < 0.01$.

3.2.10 H69 SCLC cells and culture medium induce increased IL-10 production.

TGF-β and IL-10 are potent immunosuppressive cytokines and play a key role in tumour pathogenesis (191, 192). They induce $CD4^+CD25^+$ regulatory T (Treg) cells that can suppress activation and proliferation of effector T cells (193-195). To investigate whether the suppressed $CD4^+$ T cell proliferation was accompanied by changes in TGF-β1 or IL-10 release, ELISAs were performed on the supernatants of activated $CD4^+$ T cells co-cultured with different ratios of H69 cells.

TGF-β1 secretion by H69 cells in serum-free conditioned medium was at low levels (Active: 29.53 ± 2.67 pg/ml, Latent: 75.66 ± 2.89 pg/ml, mean of n=5 experiments ± SEM) (Fig. 3.10A). However, TGF-β1 levels in the supernatants from co-culture of H69 SCLC cells with activated $CD4^+$ T cells were below the detection limit. The lack of detection of TGF-β1 in co-culture may be due to consumption by the $CD4^+$ T cells upon activation, suggesting that there may be an interaction between $CD4^+$ T cells and tumour cells.

Non-stimulated $CD4^+$ T cells or H69 cells did not produce IL-10 in the cell culture (Fig. 3.10B). However, activated $CD4^+$ T cells co-cultured with H69 SCLC cells produced significantly greater amounts of IL-10 in a dose-dependent fashion compared to activated $CD4^+$ T cells cultured alone (Fig. 3.10B). In addition, replacement of 20-40% of culture medium with H69, but not H510, cell culture supernatant also significantly increased IL-10 secretion in $CD4^+$ T cell cultures (Fig. 3.10C). The data suggest that the soluble factors produced by H69 SCLC cells can induce increased IL-10 production from activated $CD4^+$ T cells contributing to Treg cell population and suppression of $CD4^+$ T cell proliferation. These factors may include TGF-β1.

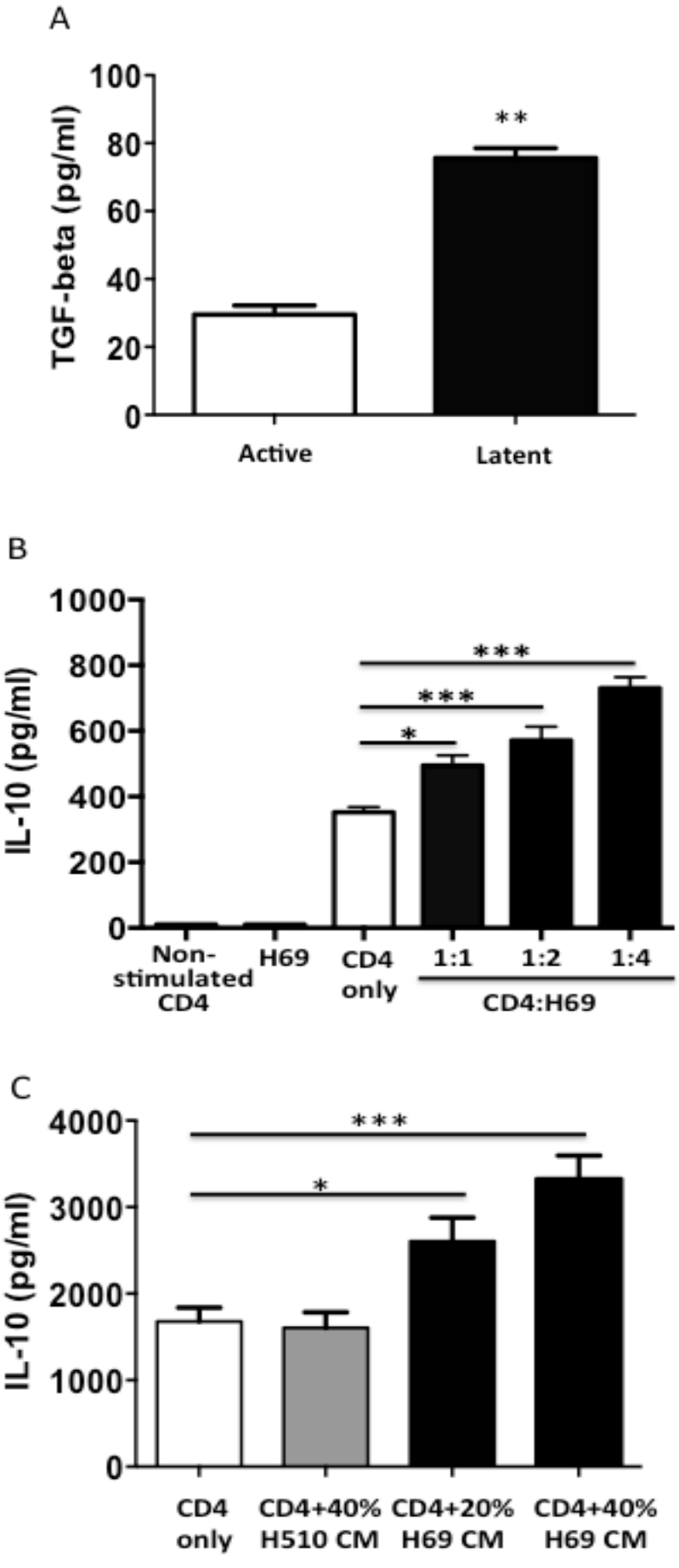

A
TGF-beta (pg/ml)
100
80
60
40
20
0
**
Active
Latent
B
IL-10 (pg/ml)
1000
800
600
400
200
0
***
***
*
Non-stimulated CD4
H69
CD4 only
1:1
1:2
1:4
CD4:H69
C
IL-10 (pg/ml)
4000
3000
2000
1000
0
***
*
CD4 only
CD4+40% H510 CM
CD4+20% H69 CM
CD4+40% H69 CM

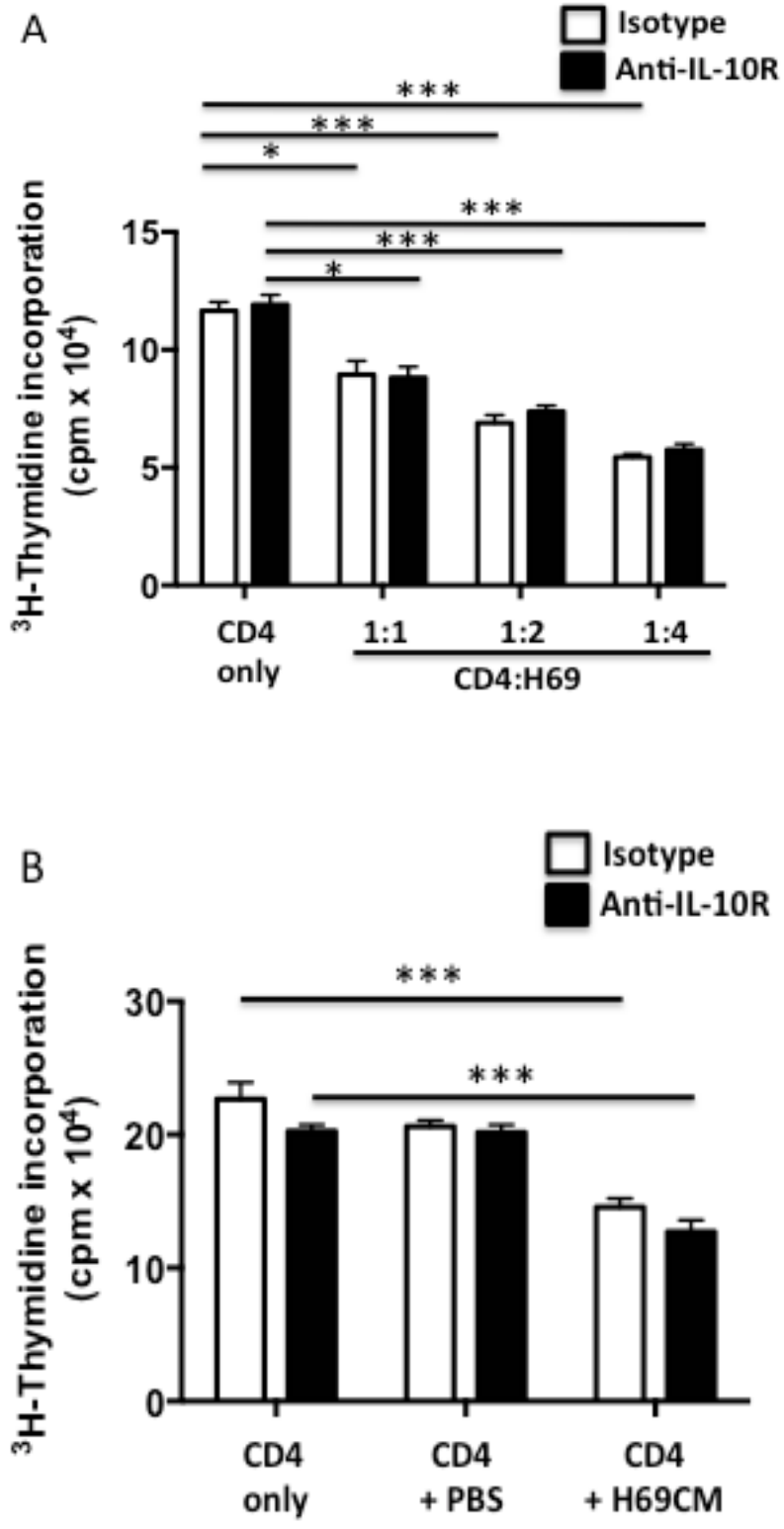
Figure 3.10: H69 SCLC cells and culture medium induce increased IL-10 production. Cytokine production was measured by ELISAs. (A) The active and latent TGF-β1 concentration in H69CM assessed by ELISA (mean ± SEM, n=5 experiments). ** Indicates $p < 0.01$. (B) The concentration of IL-10 released by $CD4^+$ T cells in the supernatants assessed by ELISA (mean ± SEM, n=6 experiments). * Indicates $p < 0.05$ and *** $p < 0.0001$. (C) The concentration of IL-10 released by $CD4^+$ T cells in the supernatants (mean ± SEM, n=6 experiments). * Indicates $p < 0.05$ and *** $p < 0.0001$.

3.2.11 Suppression of $CD4^+$ T cells by H69 cells is independent of both IL-10 and TGF-β.

To determine whether increased production of IL-10 directly contributes to the suppression of activated $CD4^+$ T cell proliferation, the effects of IL-10 function were neutralized using an anti-IL-10 receptor blocking antibody. This intervention did not affect the suppressed proliferation of activated $CD4^+$ T cells mediated by H69 SCLC cells (Fig. 3.11A). Similarly, anti-IL-10 receptor blocking antibody did not reverse the inhibition of activated $CD4^+$ T cell proliferation mediated by H69 culture supernatant (Fig. 3.11B). The data therefore indicate that although H69 SCLC cells secrete factors that both increase IL-10 secretion and suppress $CD4^+$ T cell proliferation, IL-10 is not essential for suppression of proliferation.

Similarly, to assess whether TGF-β secretion by H69 SCLC cells was responsible for the suppression of $CD4^+$ T cell proliferation, the effect of a TGF-β neutralizing antibody was assessed. Anti-TGF-β neutralizing antibody did not

abrogate the suppression of $CD4^+$ T cell proliferation mediated by H69 culture supernatant (Fig. 3.11C). The data suggest that the suppressive effect on $CD4^+$ T cell proliferation is TGF-β independent.



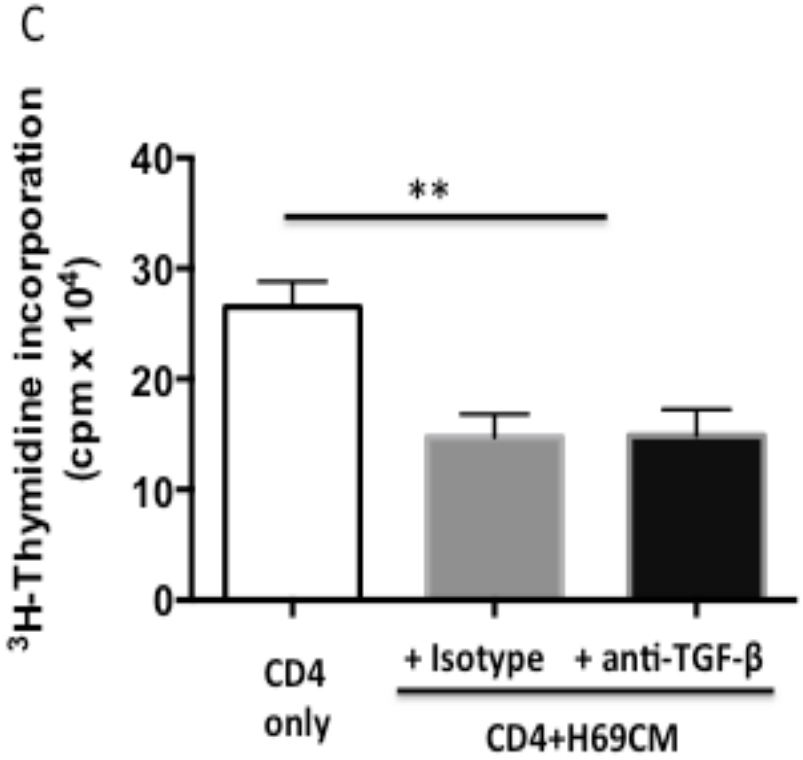


Figure 3.11: Suppression of $CD4^+$ T cells by H69 cells is independent of both IL-10 and TGF-β. An anti-IL-10 receptor blocking antibody or a TGF-β neutralizing antibody or isotype control antibodies were added to $CD4^+$ T cell culture. (A) Uptake of ^{3}H-thymidine (counts per minute; cpm) by $CD4^+$ T cells co-cultured with H69 cells in the presence of an anti-IL-10 receptor blocking antibody or isotype control (mean ± SEM, n=3 experiments). * Indicates $p < 0.05$ and *** $p < 0.0001$. (B) Uptake of ^{3}H-thymidine by $CD4^+$ T cells cultured with 40% H69CM in the presence of an anti-IL-10 receptor blocking antibody or isotype control (mean ± SEM, n=3 experiments). *** Indicates $p < 0.0001$. (C) Uptake of ^{3}H-thymidine by $CD4^+$ T cells cultured with 40% H69CM in the presence of a TGF-β neutralizing antibody or isotype control (mean ± SEM, n=3 experiments). ** Indicates $p < 0.01$.

3.3 Discussion

The local host immune response controls tumour growth and eradicates malignant cells, and thus has been employed to develop novel anti-cancer therapies. However, previous studies have suggested that SCLC cells can suppress cell-mediated immune reactions (162,165,173). $CD4^+$ T cells play central roles in anti-tumour immune responses (174, 184). I have therefore studied the effect of three different SCLC cell lines upon $CD4^+$ T cell mediated PBMC behaviour in MLR assays and established unsegregated and segregated $CD4^+$ T cell:SCLC cell co-culture systems. These have allowed me to assess whether SCLC cells could affect local immune cell behaviour and, if so, to dissect mechanistic factors.

The data in this chapter demonstrate that H69 and H345 SCLC cells can inhibit the proliferation of an MLR whilst H510 cannot. This suggests that SCLC cell lines derived from different patients specifically suppress cell-mediated immune responses in leukocytes derived from healthy donors to varying degrees. Therefore the differences in cellular immune responses observed in SCLC patients *in vivo* may be mediated by tumour cell-derived factors and not solely arise from differences in the pre-existing host immunity. The data in this chapter further demonstrate that H69 cells suppress $CD4^+$ T cell proliferation in a dose dependent fashion whilst H510 cells have no suppressive effect. H345 cells inhibit $CD4^+$ T cell proliferation only at a higher ratio (CD4:H345 1:4), consistent with a general trend of an intermediate level of immunosuppressive effects between those H69 and H510 cells.

H69 cell culture supernatant inhibits $CD4^+$ T cell proliferation in response to both T cell receptor mediated activation and mitogenic stimulation, indicating that the suppressive effect is independent of stimulation mode. H69 SCLC cells inhibit $CD4^+$ T cell proliferation by preventing activated $CD4^+$ T cells from entering the cell cycle (Fig. 3.8). Such suppression does not depend upon cell-contact and appears predominantly mediated by soluble factors (Fig. 3.4 and 3.5). The data indicate the secreted soluble factors are <30kDa in size and heat stable. Small and heat stable cytokines are therefore candidate mediators of the suppressive effect. Together these findings represent a mechanism by which SCLC cells evade or subvert cell-mediated immunity through secreting soluble molecules to suppress $CD4^+$ T cell proliferation in response to stimulation.

IL-2 is an important stimulus to $CD4^+$ T cell proliferation and is secreted in response to activation (187, 188). Previous studies demonstrated that the significantly reduced proliferative ability of tumour-infiltrating lymphocytes was associated with reduction of IL-2 release in other tumours (196,197). In SCLC, peripheral blood lymphocytes from patients have been shown to be less proliferative with reduced secretion of IL-2 in response to stimulation, and decreased IL-2 production correlates with poor survival (162,163). The data in this chapter indicate that H69 SCLC cell mediated suppression of $CD4^+$ T cell proliferation is not associated with reduction of IL-2 secretion, suggesting that the mechanism of suppression is independent of IL-2 release.

Furthermore, H69 SCLC cells do not affect IFN-γ or IL-4 secreted by $CD4^+$ T cells, but reduce IL-17 secretion that is a production of Th17 cells, suggesting

that SCLC cells may suppress a Th17 response. Previous studies have demonstrated that Th17 cells are present in the tumour microenvironment in various human cancers (198, 199). However, the effect of Th17 cells in tumours is pleiotropic. They can either promote protective anti-tumour immune responses (200-202) or suppress immune system and contribute to tumour growth (203-205). The effect of IL-17 in SCLC pathogenesis is therefore interesting and merits further investigation.

TGF-β and IL-10 are potent immunosuppressive cytokines that have molecular weight less than 30kDa and play a key role in tumour pathogenesis (191,192). They can be produced by tumour cells and have been shown to impair the proliferation, cytokine production and migratory capacities of effector T cells, suppress the functions of antigen presenting cells, and induce regulatory T (Treg) cells (206-208). TGF-β is detected at low levels in both active and latent forms from H69 culture supernatant. However, the level of TGF-β in the supernatant from co-culture of H69 cells with activated $CD4^+$ T cells is below the detection limit (<31.25 pg/ml), suggesting that TGF-β may be consumed by the T cells. Interestingly, H69 SCLC cells do not produce IL-10, but IL-10 secretion is significantly increased by co-culture of activated $CD4^+$ T cells with H69 cells in a dose-dependent fashion or with H69 culture supernatant. Therefore H69 SCLC cells may suppress cell-mediated immune responses may be through the release of TGF-β or by induction of IL-10 secretion from activated $CD4^+$ T cells to inhibit further $CD4^+$ T cell proliferation.

Previously published data demonstrated that IL-10 and TGF-β can directly inhibit human T cell proliferation through interruption of IL-2 and IFN-γ production (209, 210). However, addition of excess anti-IL-10 receptor blocking antibody (20 μg/ml, sufficient to block the effects of 10 ng/ml IL-10 activity) or anti-TGF-β antibody (10 μg/ml, sufficient to neutralize the effect of 10 ng/ml TGF-β) fails to abrogate the suppressive effect on $CD4^+$ T cell proliferation. The lack of effects of the anti-IL-10 receptor blocking antibody and the TGF-β neutralizing antibody on the suppression of $CD4^+$ T cell proliferation, may therefore indicate that neither IL-10 nor TGF-β are likely to be directly responsible for the inhibition of proliferation mediated by H69 SCLC cells. The next set of experiments focused on identification of novel soluble factors that can inhibit $CD4^+$ T cell proliferation. In addition, although IL-10 and TGF-β are not involved in direct inhibition of T cell proliferation, they have been shown to induce $CD4^+CD25^+$ regulatory T (Treg) cells that supress activation and proliferation of effector T cells (193-195). Suppression of $CD4^+$ T cell proliferation can result from expansion of Treg cells. It is therefore also important to address whether H69 cells suppress cell-mediated immune responses by inducing expansion of Treg cells.

Chapter 4: Production of IL-15 by Small Cell Lung Cancer Cells Can Induce Population of a Functional Regulatory T cell Phenotype.

Chapter 4 Production of IL-15 by Small Cell Lung Cancer Cells Can Induce Population of a Functional Regulatory T cell Phenotype

4.1 Introduction

In addition to effector immune cells, regulatory T cells are indispensable for the maintenance of dominant self tolerance and immune homeostasis (211). $CD4^+$ regulatory T cells (Tregs) are characterized by functional ability to suppress the proliferation of conventional $CD4^+$ T cells and by the expression of the cell surface marker CD25 and the intracellular marker (FoxP3) (212). Naturally occurring Tregs (nTregs), which are developed in thymus, comprise 5-10% of peripheral $CD4^+$ T cells (213). They exert suppressive activity on effector T cells through a cell-to-cell contact dependent, IL-10 and TGF-β independent mechanism (214). Adaptively induced Tregs (iTregs) are induced from conventional $CD4^+$ T cells in the periphery in a tolerogeneic environment. The suppressive effect of iTregs is usually mediated by secreting large amounts of the potent immunosuppressive cytokines IL-10 and TGF-β (214).

In the past years, immune therapies have been introduced as treatments for a variety of cancers (175). However, $CD4^+FoxP3^+$ Treg cells are believed to be one of immunosuppressive elements present in cancer patients that are critical impediments to the success of cancer immunotherapy (175, 176). Increased numbers of $FoxP3^+$ Treg cells were first found in patients with NSCLC and ovarian cancer (215). Subsequently, a higher frequency of Tregs in peripheral blood has been reported in the patients with various cancers including NSCLC, ovarian cancer, breast cancer, gastric cancer, esophageal cancer, lymphoma, leukaemia, melanoma and pancreatic cancer (215-219). These $FoxP3^+$ cells isolated from peripheral blood or solid tumours of patients have been shown to

have suppressive effects *in vitro* and may thus contribute to tumour growth (220, 221). There are different compositions of $FoxP3^+$ cell subpopulations with distinct phenotypes and functions present in tumour tissues (176, 222). Accumulation of suppressive $FoxP3^+$ Treg cells can promote tumour cell growth and thus predict a poor prognosis, whilst non-suppressive $FoxP3^+$ cells, which are capable of releasing pro-inflammatory cytokines, can activate anti-tumour immune responses and thus contribute to better survival in cancer patients (222). The role of Treg cells in cancer is not fully understood and differs with type of tumours. Therefore, determining the effects of Treg cells on initiation and progression of SCLC may help with the development of novel immunotherapeutic strategies.

There are several potential sources for Treg cells in the tumour microenvironment. Treg cells express CC-chemokine receptor 4 (CCR4). Tumour cells and/or tumour infiltrating antigen-presenting cells (APCs) produce the CC-chemokine ligand 22 (CCL22), which can chemo-attract and recruit Treg cells in peripheral blood or lymph nodes to infiltrate into tumour sites (223). In addition, the tumour microenvironment contains factors such as VEGF, IL-10 and TGF-β that suppress APC differentiation and function. Dysfunctional APCs can induce Treg cell differentiation and expansion (220). Furthermore, effector $CD4^+$ T cells can be converted into Treg cells by the presence of high levels of IL-10 and TGF-β present in the tumour microenvironment (224, 225). Therefore, increased frequencies of $FoxP3^+$ Treg cells that are found in the tumour microenvironment may contain recruited and expanded nTregs, and/or locally converted iTregs.

Phenotypic characterization of Tregs is difficult due to lack of specific markers. Under physiological conditions, CD25 is constitutive expressed on the cell surface of Tregs, but has poor specificity under conditions of immune activation. FoxP3 has been identified as a master transcriptional regulator in the development and suppressive function of Tregs, but there is increasing evidence showing that $CD4^+CD25^-$ T cells become FoxP3-positive upon activation (226, 227). CD127, the α-chain of the IL-7 receptor, has been shown to be a marker to discriminate between human CD4 regulatory and activated T cells, and its expression inversely correlates with FOXP3 and suppressive function (228, 229). Helios protein, a member of Ikaros transcription factor family, was first reported to distinguish between thymic-derived nTregs and peripherally induced iTregs (230). However, it is no longer a useful marker for identification of Treg cell phenotype. Helios is associated with T cell activation and proliferation (231), and it is selectively up-regulated in $CD4^+$ T cells during differentiation of Th2 and follicular helper T (Tfh) cells responses *in vivo* independently of FoxP3 expression (232). It has been described that Helios is also expressed in peripherally induced $FoxP3^+$ Tregs both *in vitro* and *in vivo* (233).

In this chapter an *in vitro* co-culture of H69 SCLC cells with $CD4^+$ T cells was established to explore the hypothesis that SCLC cells induce increased population of $FoxP3^+$ Treg cells and if so to evaluate their phenotype and function.

4.2 Results

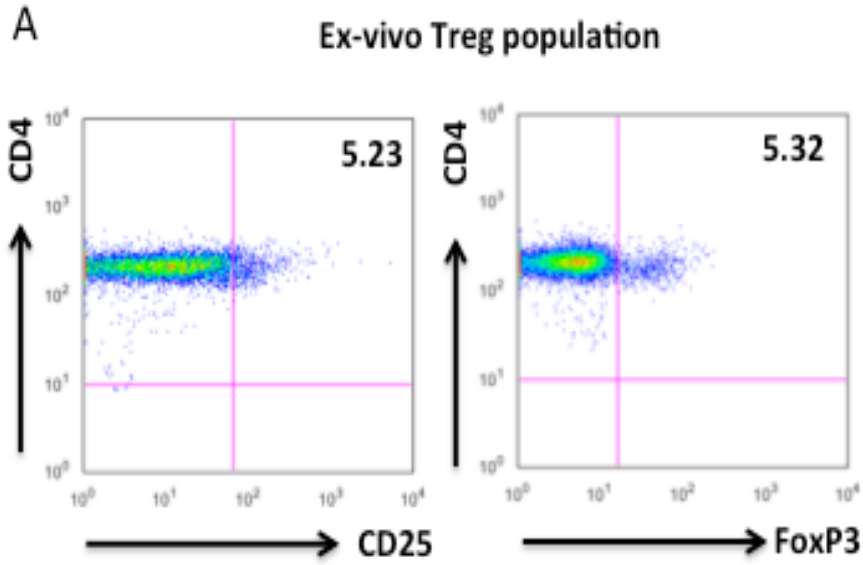
4.2.1 SCLC cell-secreted soluble molecules induce $CD4^+CD25^+CD127^{low}Helios^-FoxP3^+$ Treg cells in a dose dependent fashion.

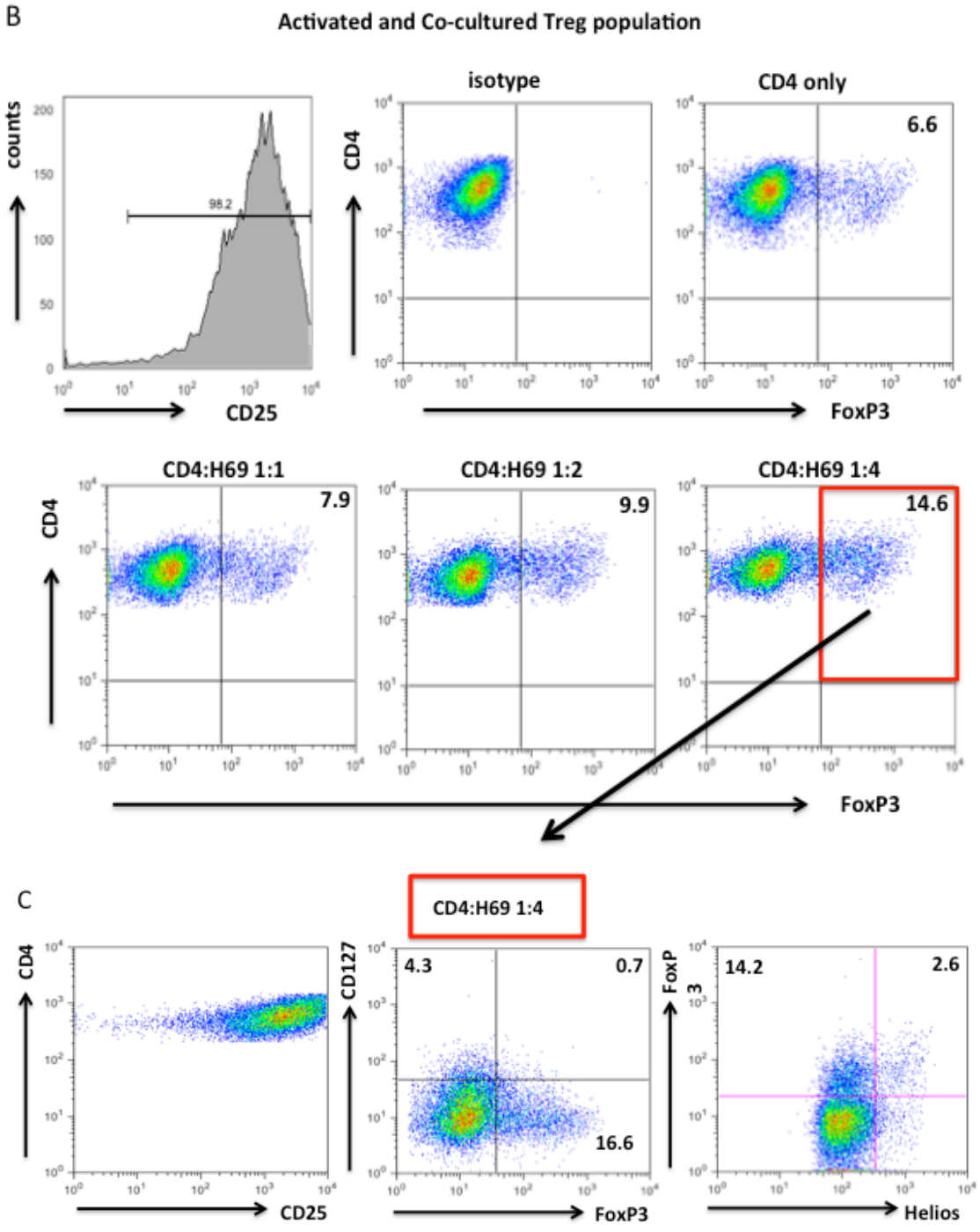
The observed suppression of activated $CD4^+$ T cell proliferation by H69 SCLC cells or H69 culture supernatant was associated with increased IL-10 concentration in the supernatant (Chapter 3). I therefore went on to investigate whether there were also alterations in Treg population that could result in both increased IL-10 secretion and the suppression of $CD4^+$ T cell proliferation.

Surface markers CD4 and CD25, and the intracellular marker FoxP3 were used to identify Tregs. Firstly, the frequency of $CD4^+CD25^+FoxP3^+$ Tregs in peripheral blood was found to be 5.42 ± 0.22 % of total purified $CD4^+$ T cells (Fig. 4.1A and 4.1D). Above 98% of $CD4^+$ T cells were activated in response to anti-CD3/CD28 stimulation, and the cells were gated on CD25 expression (Fig. 4.1B).

Co-culture of H69 SCLC cells in transwells with activated $CD4^+$ T cells significantly induced the expansion of $CD4^+CD25^+FoxP3^+$ Treg cells in a dose-dependent fashion in comparison with activated $CD4^+$ T cells cultured alone (Fig. 4.1B and 4.1D). To further identify the phenotype of this increased Treg population, the cells were stained with anti-CD4, anti-CD25, anti-CD127, anti-Helios and anti-FoxP3 antibodies, and were gated on CD4, CD25 and FoxP3 expression. As shown, H69 SCLC cells-induced expansion of Treg cells had a phenotype of $CD4^+CD25^+CD127^{low}Helios^-FoxP3^+$ (Fig. 4.1C).

Similarly, the frequency of $CD4^+CD25^+FoxP3^+$ cells was also significantly increased by culture with H69CM compared to $CD4^+$ T cells cultured alone following activation (Fig. 4.1E). These data suggest that H69 SCLC cells constitutively produce soluble factors that induce the expansion of $CD4^+CD25^+FoxP3^+$ Treg cells, and so suppress $CD4^+$ T cell proliferation.




B
Activated and Co-cultured Treg population
counts
98.2
CD25
isotype
CD4
CD4 only
6.6
FoxP3
CD4:H69 1:1
7.9
CD4:H69 1:2
9.9
CD4:H69 1:4
14.6
FoxP3
C
CD4:H69 1:4
CD4
CD25
CD127
4.3
0.7
16.6
FoxP3
FoxP 3
14.2
2.6
Helios

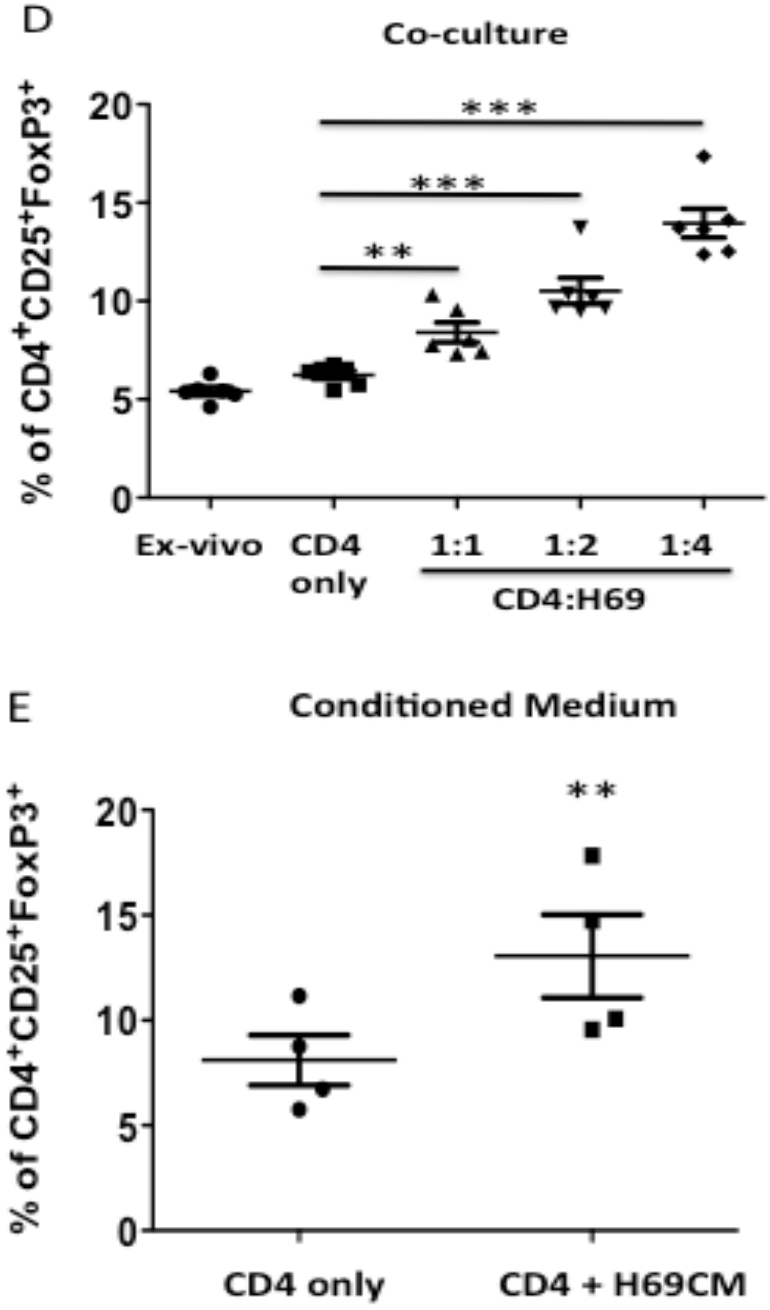


Figure 4.1: Soluble molecules secreted by H69 SCLC cells induce FoxP3$^+$ Treg cells in a dose dependent fashion. The experiments were performed as described in Chapter 2. (A) Representative flow cytometry data showing the percentages of *ex vivo* CD4$^+$CD25$^+$ and CD4$^+$FoxP3$^+$ cells from peripheral blood. (B) Representative flow cytometry data showing the populations of CD4$^+$CD25$^+$FoxP3$^+$ cells. (C) Representative flow cytometry data showing the populations of CD4$^+$CD25$^+$CD127^{low}Helios$^-$FoxP3$^+$ cells. (D) The populations of CD4$^+$CD25$^+$FoxP3$^+$ cells from *ex vivo*, activated CD4$^+$ T cells cultured alone or co-cultured with

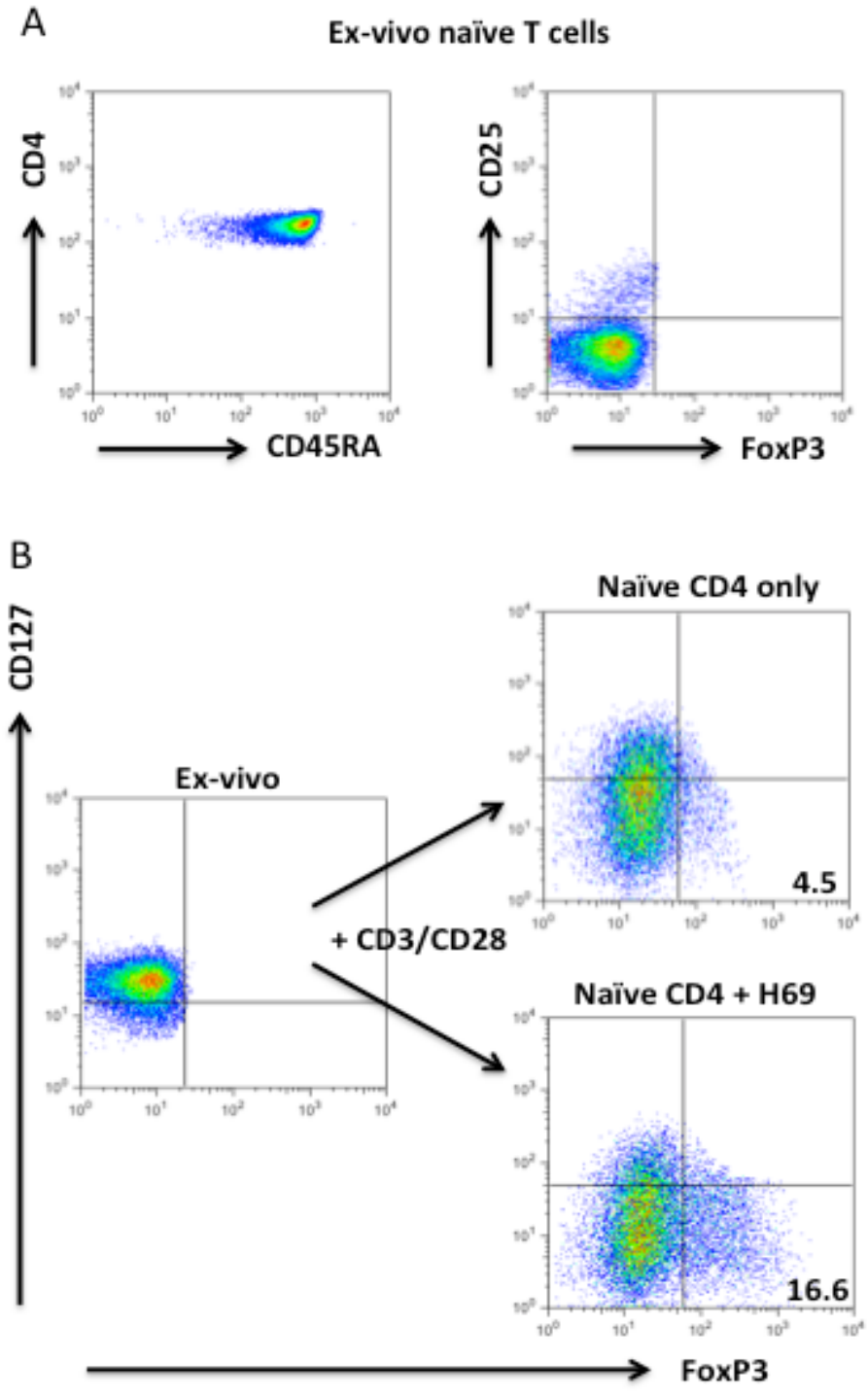
different ratios of H69 cells (mean ± SEM, n=6 experiments). ** Indicates $p < 0.01$ and *** $p < 0.0001$. (E) The populations of $CD4^+CD25^+FoxP3^+$ cells from activated $CD4^+$ T cells cultured alone or with 40% of H69 SCLC CM (mean ± SEM, n=4 experiments). ** Indicates $p < 0.01$.

4.2.2 H69 SCLC cells induce increased Treg population through the differentiation of naïve $CD4^+$ T cells.

It remains difficult to accurately distinguish iTregs from nTregs using current molecular markers. Both express the canonical Treg markers: CD25, FoxP3, CTLA-4 and GITR, although nTregs exhibit a higher expression of programmed cell death-1 (PD-1), CD73, neuropilin 1 (Nrp1) and Helios compared with iTreg (234), there is no consistent or reliable marker.

To assess whether the increased population of $FoxP3^+$ Tregs was due to expansion of pre-existing nTregs or induction from naïve T cells (iTregs), naïve $CD4^+$ T cells were first purified. The purified naïve T cells had the phenotype of $CD4^+CD45RA^+CD25^-CD127^{Hi}FoxP3^-$ (Fig. 4.2A), confirming the absence of pre-existing nTregs. Subsequent co-culture of H69 SCLC cells with naïve $CD4^+$ T cells significantly increased $CD25^+CD127^{low}FoxP3^+$ Treg cell population compared to naïve $CD4^+$ T cells cultured alone in response to stimulation (Naïve CD4 only: 4.5%, Naïve CD4+H69: 16.6%, Fig. 4.2B). The result was highly similar to the increase in Treg population induced from a mixed population of $CD4^+$ T cells by H69 SCLC cells (Fig. 4.1B, 4.1C and 4.1D). Furthermore, these H69 SCLC cell-induced $FoxP3^+$ cells showed impaired proliferative ability (Fig.

4.2C). Together these data suggest that the increased FoxP3^{+} Treg cell population was derived from differentiation of naïve CD4^{+} T cells rather than by expansion of pre-existing nTregs.



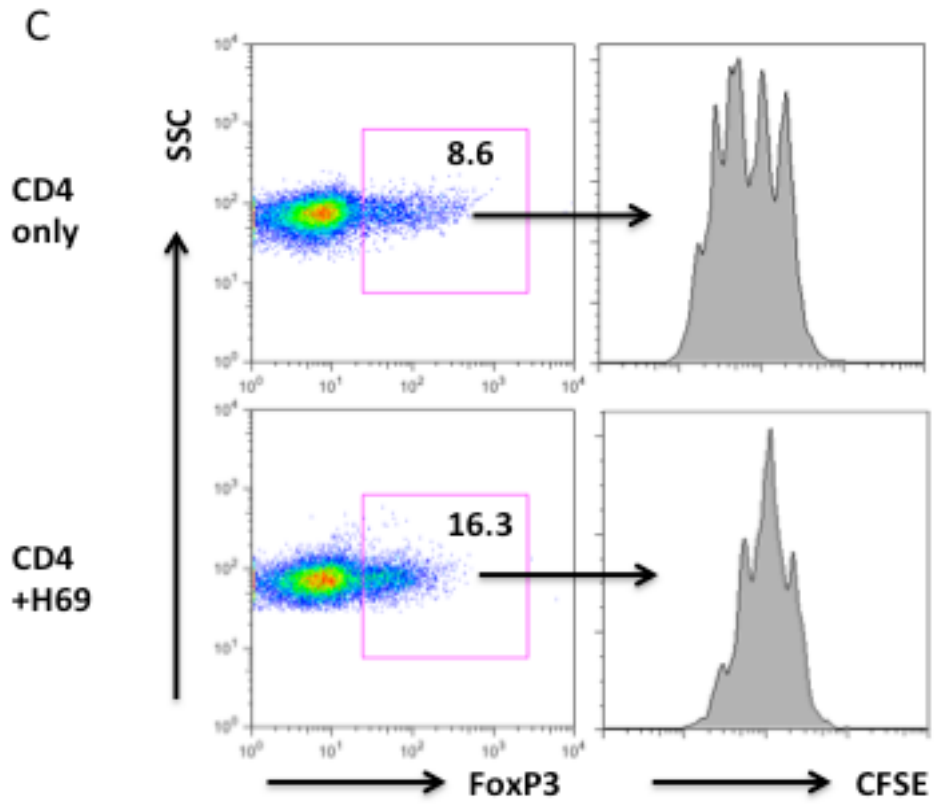


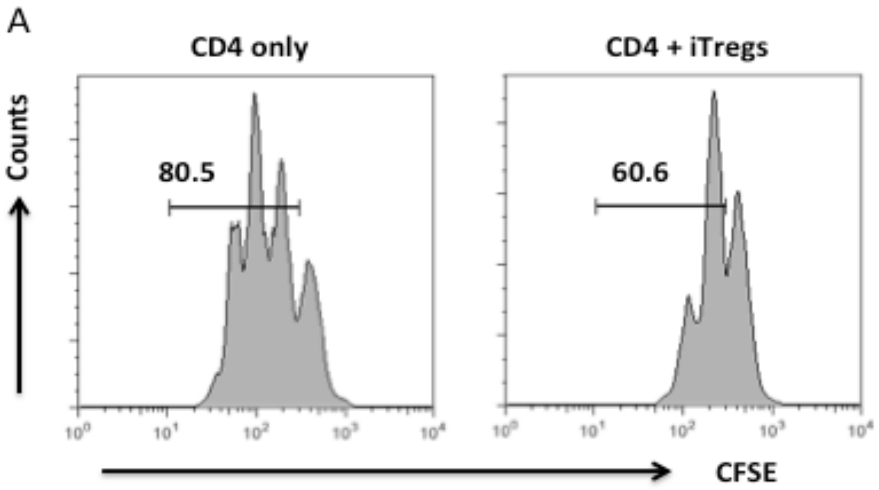
Figure 4.2: SCLC-induced Treg cells are derived from differentiation of naïve $CD4^+$ T cells. Representative flow cytometry showing (A) the populations of purified naïve $CD4^+CD45RA^+FoxP3^-$ cells (B) the populations of $CD4^+CD25^+CD127^{low}FoxP3^+$ cells in the absence and presence of H69 cells (C) the proliferations of $FoxP3^+$ cells in the presence or absence of H69 cells.

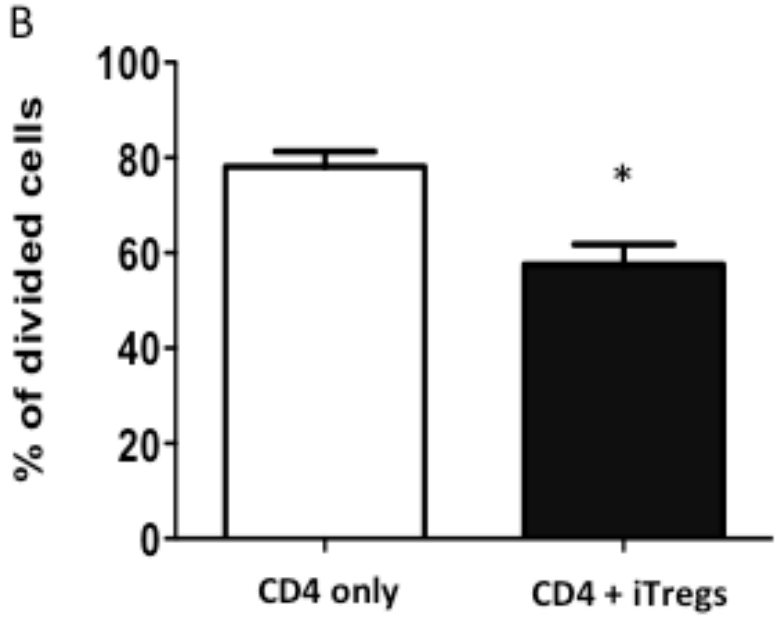
4.2.3: SCLC-induced Treg cells are functional to suppress effector $CD4^+$ T cell proliferation.

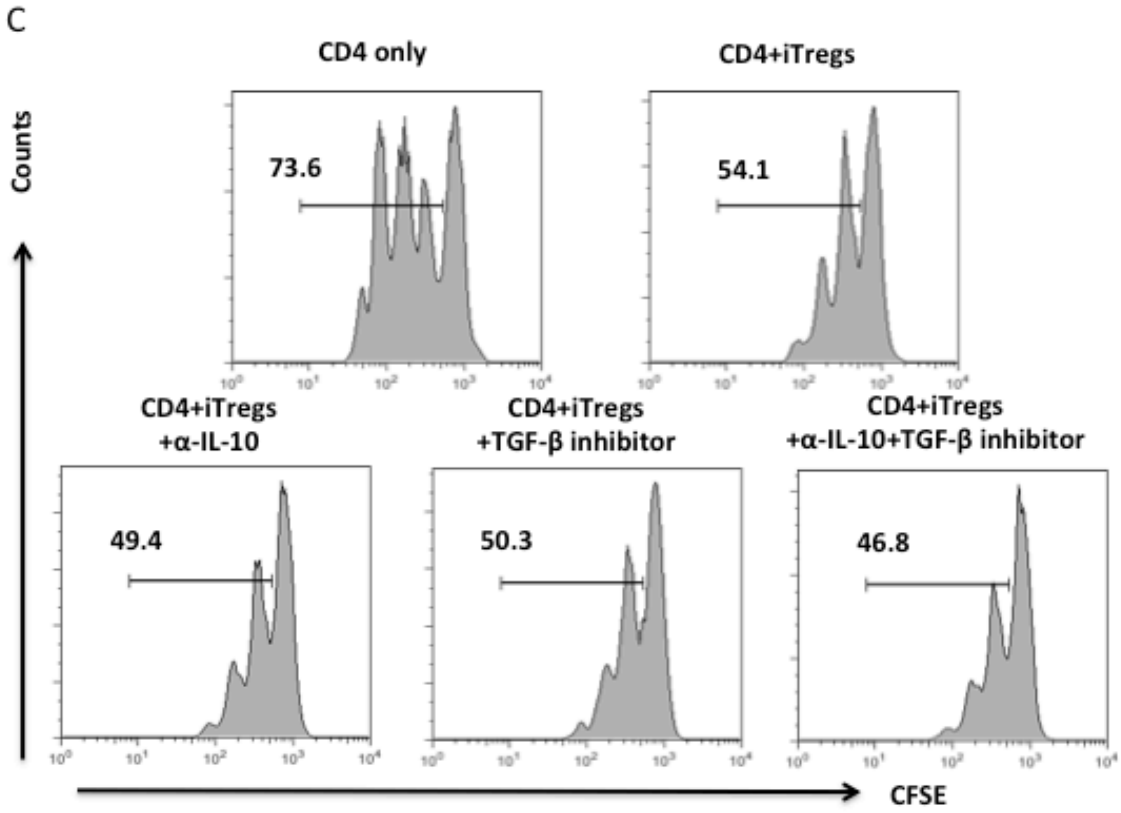
To determine whether the $FoxP3^+$ Treg population induced from naïve $CD4^+$ T cells can suppress the conventional $CD4^+$ T cell proliferation, a Treg functional assay was established. The proliferation of naïve $CD4^+$ T cells in response to stimulation was significantly suppressed by the presence of autologous $CD4^+$ T cells previously co-cultured with H69 SCLC cells (Fig. 4.3A and 4.3B). The data

suggest that the FoxP3$^+$ cells induced by H69 SCLC cells are indeed functional Treg cells capable of suppressing CD4$^+$ T cell proliferation.

To investigate whether the suppressive function of these induced Tregs is mediated by IL-10 or TGF-β secretion, anti-IL-10 receptor blocking antibody and TGF-β signalling inhibitor (SB431542) were used to block their functions. Blocking of IL-10 or TGF-β activities, or both together, failed to abrogate the suppressive effect on CD4$^+$ T cell proliferation that was mediated by H69 SCLC cell-induced FoxP3$^+$ cells (Fig. 4.3C and 4.3D). The data are consistent with observations for CD4$^+$ T cells as a whole, suggesting that the suppressive effect of H69 SCLC cell-induced Treg cells is independent of IL-10 or TGF-β immunosuppressive cytokine production.




B
% of divided cells
100
80
60
40
20
0
*
CD4 only
CD4 + iTregs


C
Counts
CD4 only
73.6
CD4+iTregs
54.1
CD4+iTregs
+α-IL-10
49.4
CD4+iTregs
+TGF-β inhibitor
50.3
CD4+iTregs
+α-IL-10+TGF-β inhibitor
46.8
CFSE

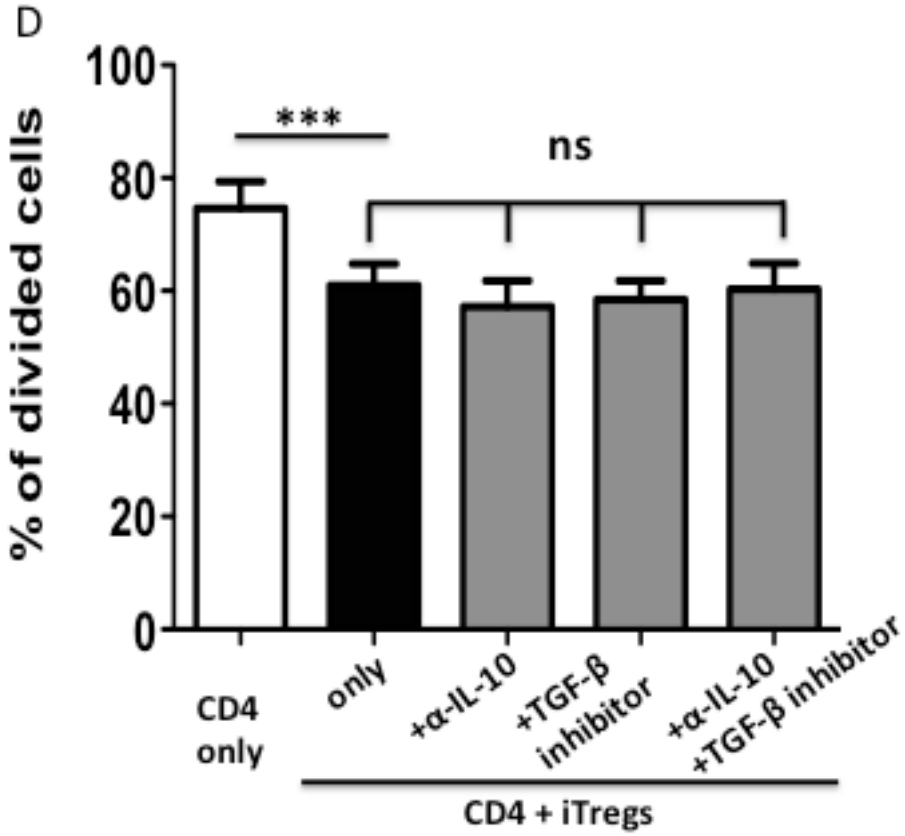


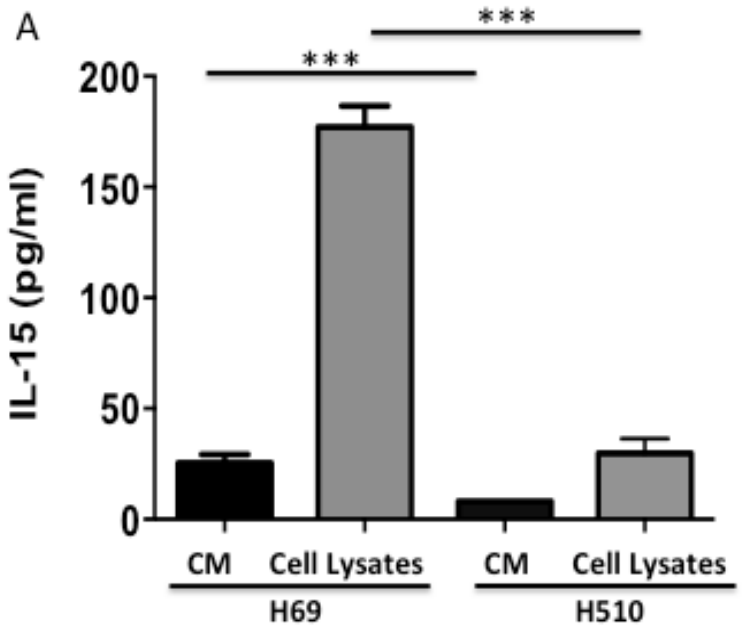
Figure 4.3: SCLC-induced Treg cells are functional to suppress effector $CD4^+$ T cell proliferation. The experiments were performed as described in section 2.17 Chapter 2. (A) Representative flow cytometry showing the total populations of dividing $CD4^+$ T cells in the absence and presence of iTregs. (B) The total populations of dividing $CD4^+$ T cells (mean ± SEM, n=4 experiments). * Indicates $p < 0.05$. (C) Representative flow cytometry showing the total populations of dividing $CD4^+$ T cells in the absence and presence of iTregs with or without the anti-IL-10 receptor antibody or the TGF-β signalling inhibitor (SB431542). (D) The populations of dividing $CD4^+$ T cells (mean ± SEM, n=3 experiments). *** Indicates $p < 0.0001$.

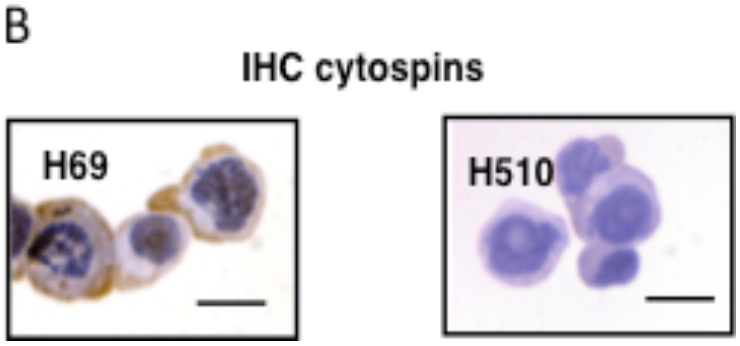
4.2.4 H69 but not H510 SCLC cells produce IL-15.

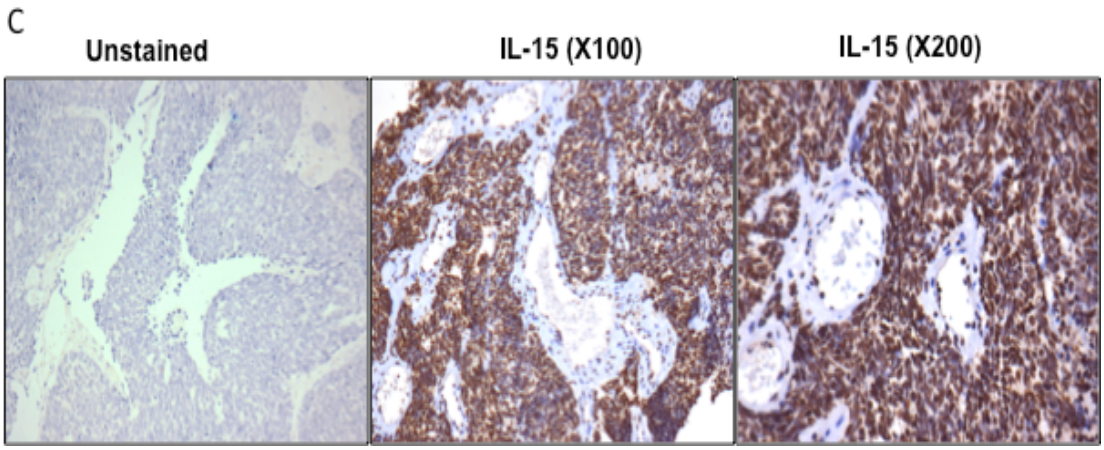
The studies presented in Chapter 3 indicated that H69 but not H510 cells secreted small (<30kDa), heat-stable soluble factors capable of inhibiting $CD4^+$

T cell proliferation (Fig. 3.2B, 3,3B, 3.5A and 3.7). I therefore compared publicly available microarray data on H69 and H510 cells (GSE7068, Gene Expression Omnibus) to identify differentially expressed cytokine genes. Genes encoding IL-1α, IL-11, IL-15, IL-16, BMP-7, CSF-2 and TGF-β2 were identified as upregulated in H69 cells relative to H510 cells. Of these TGF-β (193) and IL-15 (235, 236) signalling have previously been described in relation to induction of Treg cells. TGF-β had no effect on suppression of $CD4^+$ T cell proliferation in my experimental system (Fig. 3.11C). I therefore went on to study IL-15 as a candidate mediator of Treg cell induction.

IL-15 levels were examined in H69 and H510 cell conditioned medium and cell lysates. IL-15 was detected in H69 cell culture supernatant at a low concentration (25.5 ± 3.7 pg/ml), but was below the detection limit (< 8 pg/ml) in H510 cell culture medium (Fig. 4.4A). H69 cell lysates contained much higher levels of IL-15 protein than those from H510 cells (Fig. 4.4A). Immunostaining on cytospins showed IL-15 was present in H69 but not in H510 cells (Fig. 4.4B). Importantly, malignant cells in tumour biopsies from SCLC patients contained large amounts of IL-15 (Fig. 4.4C). Addition of either recombinant IL-15 protein or IL-15 neutralizing antibody to H69 cell culture did not have effect on H69 tumour cell growth (Fig. 4.4D).


A
***
***
200
150
100
50
0
IL-15 (pg/ml)
CM
Cell Lysates
CM
Cell Lysates
H69
H510


B
IHC cytospins
H69
H510


C
Unstained
IL-15 (X100)
IL-15 (X200)

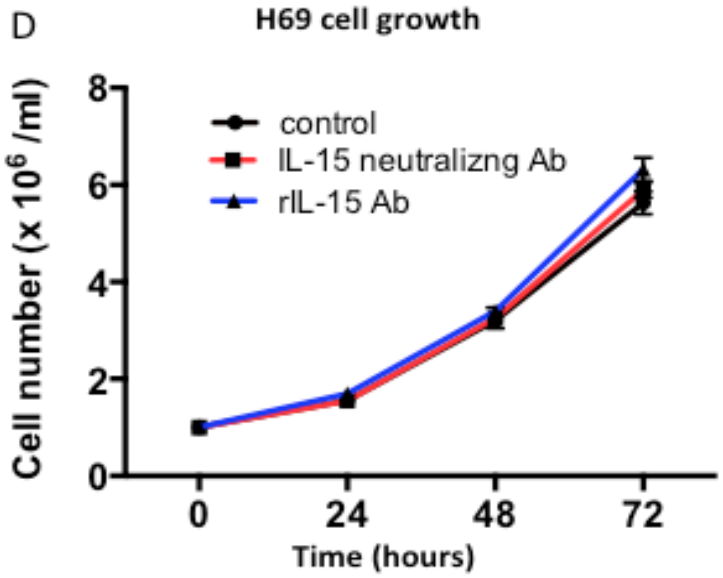
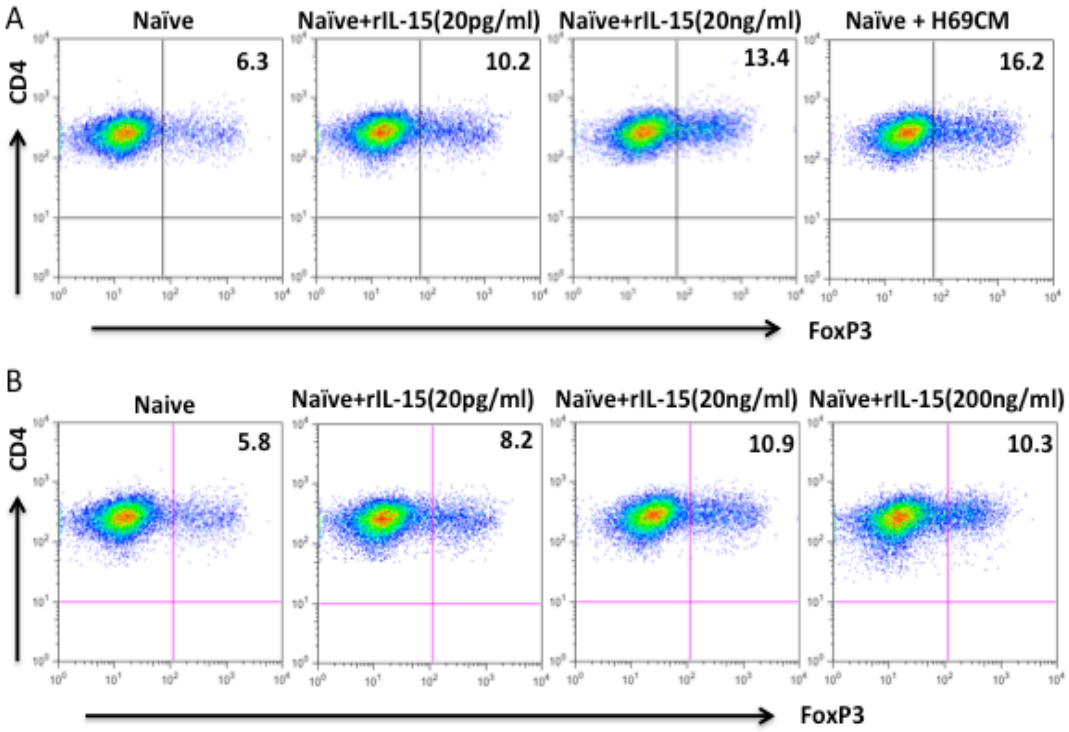


Figure 4.4: H69 but not H510 SCLC cells produce IL-15. (A) IL-15 concentration in H69 and H510 CM and cell lysates assessed by ELISA (mean ± SEM n=4 experiments). *** Indicates $p < 0.0001$. (B) Representative immunohistochemistry staining for IL-15 on H69 and H510 cell cytospins. (C) Representative immunohistochemistry staining for IL-15 on tumour biopsy sections from SCLC patients. Left panel: no IL-15 staining, middle panel: IL-15 staining under X100 magnification, right panel: IL-15 staining under X200 magnification. (D) Graph shows H69 cell numbers at 24, 48 and 72 hours when cultured alone of in the presence of recombinant IL-15 or anti-IL-15 neutralizing antibody (mean ± SEM, n=4 experiments).

4.2.5 IL-15 is a factor in H69CM that induces Treg cell population.

To investigate whether IL-15 induces Treg populations, recombinant IL-15 (rIL-15) was added to naïve $CD4^+$ T cells in response to stimulation. Addition of recombinant rIL-15 protein significantly increased the population of $CD4^+CD25^+FoxP3^+$ cells in a dose-dependent fashion compared to $CD4^+$ T cells cultured alone (Fig. 4.5A and 4.5C). There was no difference in Treg cell induction between IL-15 protein at concentrations 20ng/ml and 200ng/ml (Fig.

4.5B). Interestingly, H69 cell conditioned medium (H69CM) induced significantly greater expansion of $CD4^+CD25^+FoxP3^+$ cells in comparison with the effect of recombinant IL-15 (Fig. 4.5A and 4.5C). The data suggest that IL-15 is one but not the only soluble factor produced by H69 cells that can induce the differentiation of $CD4^+CD25^+FoxP3^+$ Treg cells from naïve $CD4^+$ T cells in response to activation. Alternatively, the rIL-15 used may be less active than endogenous IL-15 due to effects of purification process and/or post-translational modification of IL-15 *in vivo*.



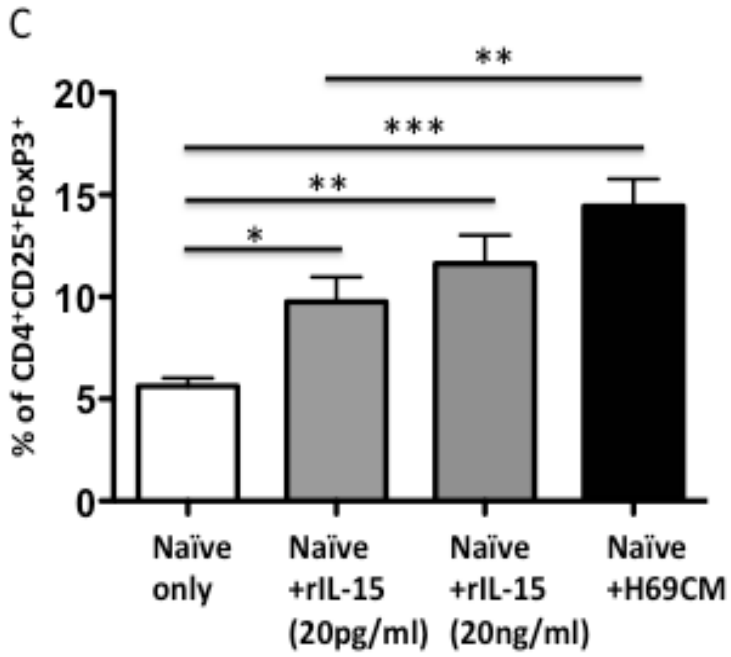


Figure 4.5: IL-15 is a factor in H69CM that induces Treg cell population. Representative flow cytometry showing the populations of $CD4^+CD25^+FoxP3^+$ cells following incubation of anti-CD3/CD28 stimulated $CD4^+$ T cells with 20pg/ml or 20ng/ml rIL-15 or 40% H69CM (A) or 200ng rIL-15 (B). (C) The percentages of $CD4^+CD25^+FoxP3^+$ cells from activated $CD4^+$ T cells cultured alone, or with 20pg/ml or 20ng/ml rIL-15 protein, or with 40% H69CM (mean ± SEM, n=3 experiments). * Indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$.

4.2.6 Addition of rIL-15 to H510CM has no additional effect on Treg cell induction.

To investigate whether addition of IL-15 to H510 cell conditioned medium (H510CM) can have the same effect on Treg cell induction as H69 culture supernatant, recombinant IL-15 protein at the concentration of 20 pg/ml was added to $CD4^+$ T cells in the presence of H510CM. Addition of IL-15 but not H510CM increased $CD4^+CD25^+FoxP3^+$ Treg cell population compared to naïve $CD4^+$ T cells cultured alone in response to activation (Fig. 4.6A and 4.6B).

Interestingly, the combination of IL-15 and H510CM did not induce greater Treg cell population compared to the effect of IL-15 alone on Treg cell induction (Fig. 4.6A and 4.6B). Taken together these data suggest that H510CM has no additional synergistic or suppressive effect on induction of Treg cell population compared to IL-15 alone. This is in contrast to the observation that rIL-15 at concentrations found in H69CM had a lesser effect on Treg cell induction than H69CM itself. Therefore, there may be other soluble factors present in H69CM that are involved in Treg cell induction that are absent in H510CM.

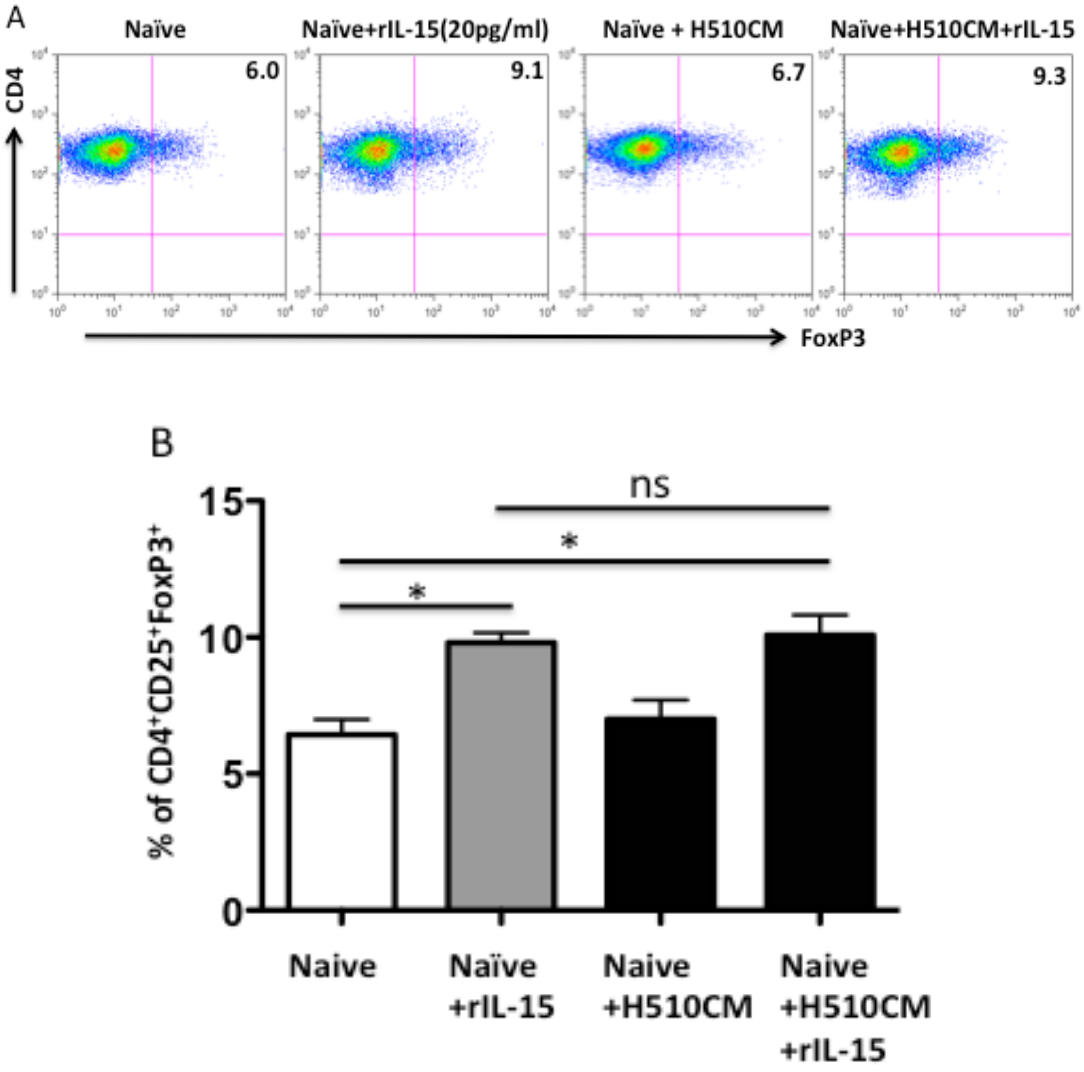
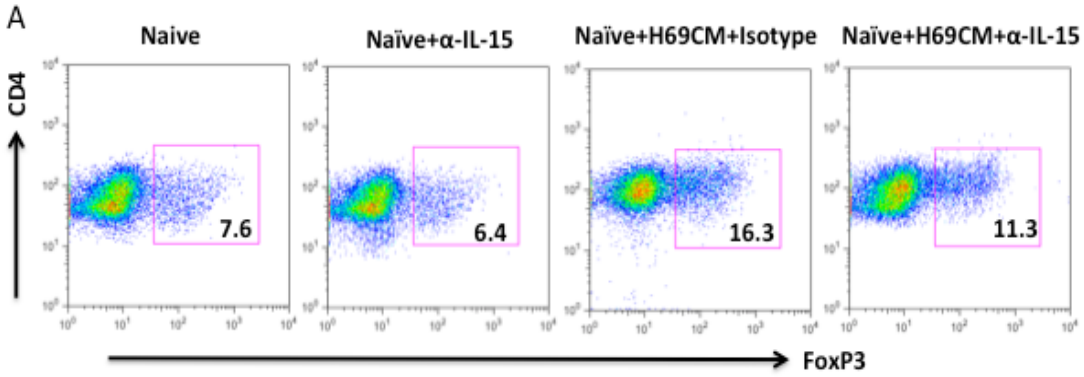


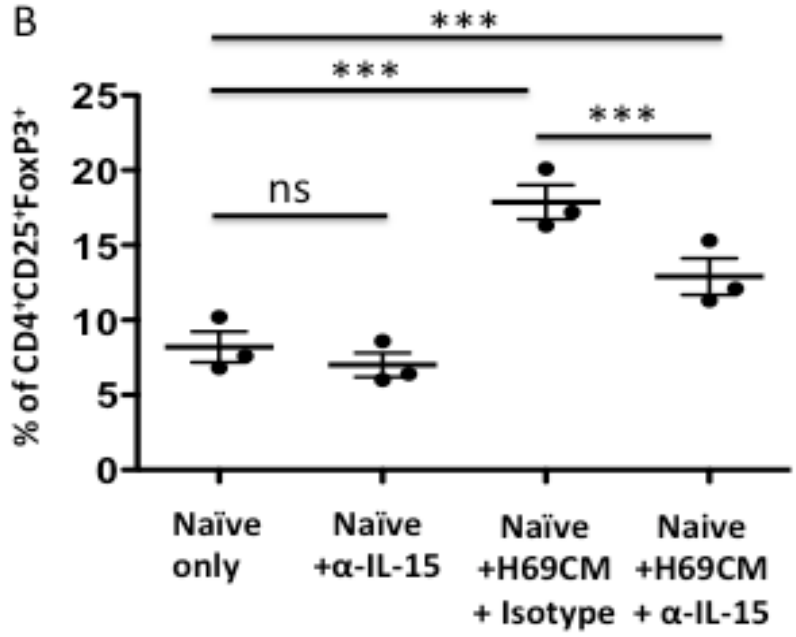
Figure 4.6: H510CM does not affect Treg cell induction by rIL-15. (A) Representative flow cytometry showing the populations of $CD4^+CD25^+FoxP3^+$ cells induced from naïve $CD4^+$ T cells by rIL-15 alone and in combination with H510CM. (B) The percentages of $CD4^+CD25^+FoxP3^+$ cells from activated $CD4^+$ T cells cultured alone, or with 20pg/ml rIL-15, or with 40% H510CM, or with rIL-15 and H510CM (mean ± SEM, n=4 experiments). * Indicates $p < 0.05$.

4.2.7 Blocking IL-15 reduces H69 SCLC cells-induced Treg cell differentiation and IL-10 secretion.

To investigate the effect of blocking IL-15 on Treg cell population and IL-10 secretion induced by H69 cells, IL-15 neutralizing antibody was used. There was no difference in Treg cell population between $CD4^+$ T cells cultured alone and cultured with IL-15 neutralizing antibody (Fig. 4.7A and 4.7B). The population of $CD4^+CD25^+FoxP3^+$ cells was significantly increased by co-culture with H69 SCLC culture medium in the presence of isotype control antibody. However, addition of IL-15 neutralizing antibody significantly reduced the expansion of Treg cells that was induced by H69CM in the presence of isotype control antibody (Fig. 4.7A and 4.7B). Interestingly, neutralization of IL-15 in H69CM failed to completely abrogate Treg induction compared to $CD4^+$ T cell cultured alone (Fig. 4.7A and 4.7B). The data suggest that inhibition of IL-15 activity partially blocks the Treg cell differentiation that is induced by H69 SCLC cells. This further supports the hypothesis that IL-15 is only one of the secreted factors responsible for iTreg induction in this SCLC model.

Similarly, IL-10 secretion was significantly reduced by addition of IL-15 neutralizing antibody to H69CM relative to isotype control antibody in this system (Fig. 4.7C). IL-10 production from activated $CD4^+$ T cells cultured with H69CM in the presence of IL-15 neutralizing antibody remained significantly higher than activated $CD4^+$ T cells cultured alone. The data suggest that blocking IL-15 activity partially reduces H69 SCLC cell-induced IL-10 secretion from activated $CD4^+$ T cells.





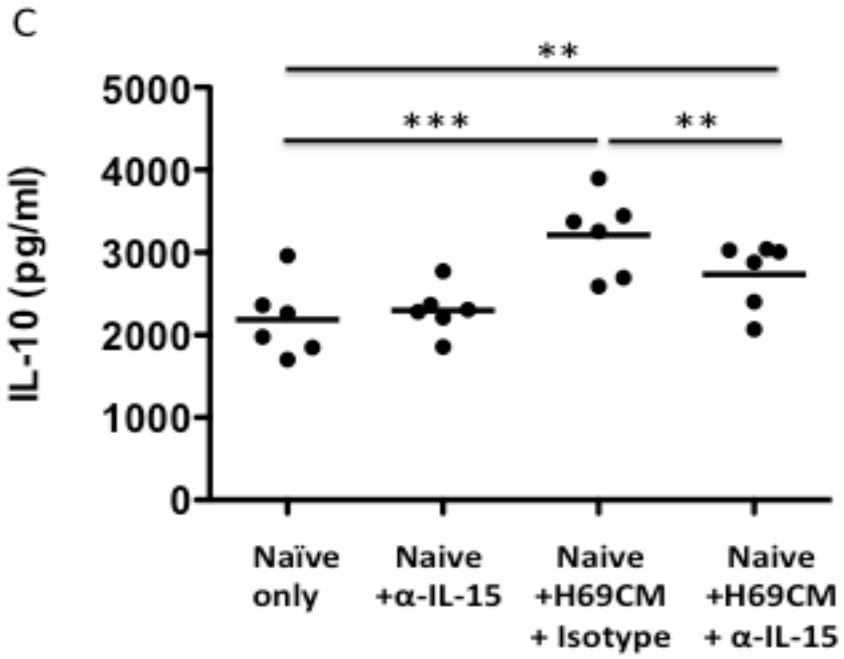


Figure 4.7: Blocking IL-15 activity reduces H69 SCLC cells-induced Treg cell differentiation and IL-10 secretion. (A) Representative flow cytometry showing the populations of $CD4^+CD25^+FoxP3^+$ cells following incubation of anti-CD3/CD28 stimulated naïve $CD4^+$ T cultured alone, or with IL-15 neutralizing antibody (α-IL-15), or with H69CM plus isotype control antibody or with H69CM plus α-IL-15. (B) The percentages of $CD4^+CD25^+FoxP3^+$ cells (mean ± SEM, n=4 experiments). *** Indicates $p < 0.0001$. (C) The concentration of IL-10 released from $CD4^+$ T cells in the supernatants (mean ± SEM, n=6 experiments). ** Indicates $p < 0.01$ and *** $p < 0.0001$.

4.2.8 Blocking of IL-15 activity partially reverses H69 SCLC cell-induced inhibition of MLR and $CD4^+$ T cell proliferation.

To investigate whether the suppression of MLR mediated by H69 SCLC cells can be reversed by blocking IL-15 activity, IL-15 neutralizing antibody was added. The ^{3}H-thymidine uptake in MLR was not affected by the addition of IL-

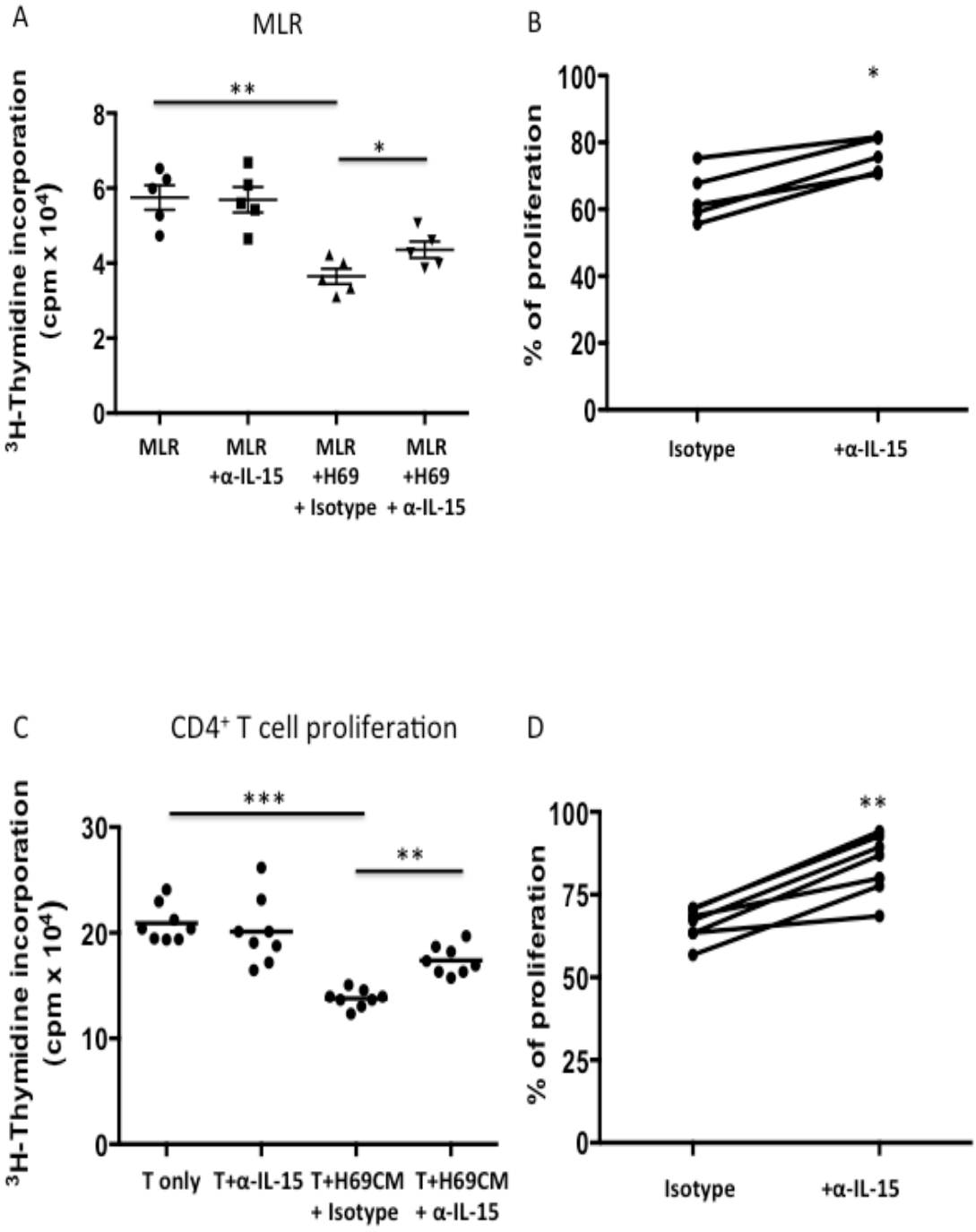
15 neutralizing antibody, but was significantly reduced by co-culture with H69 cells in the presence of isotype control antibody (Fig. 4.8A). Neutralization of IL-15 significantly reversed the suppression of MLR mediated by H69 cells (Fig. 4.8A). The percentage of MLR proliferation was suppressed to 63.8 ± 3.5% by co-culture with H69 cells, but was significantly increased to 76 ± 2.4% by neutralization of IL-15 (Fig. 4.8B). The data suggest that blocking of IL-15 activity partially reverses the inhibition of MLR proliferation.

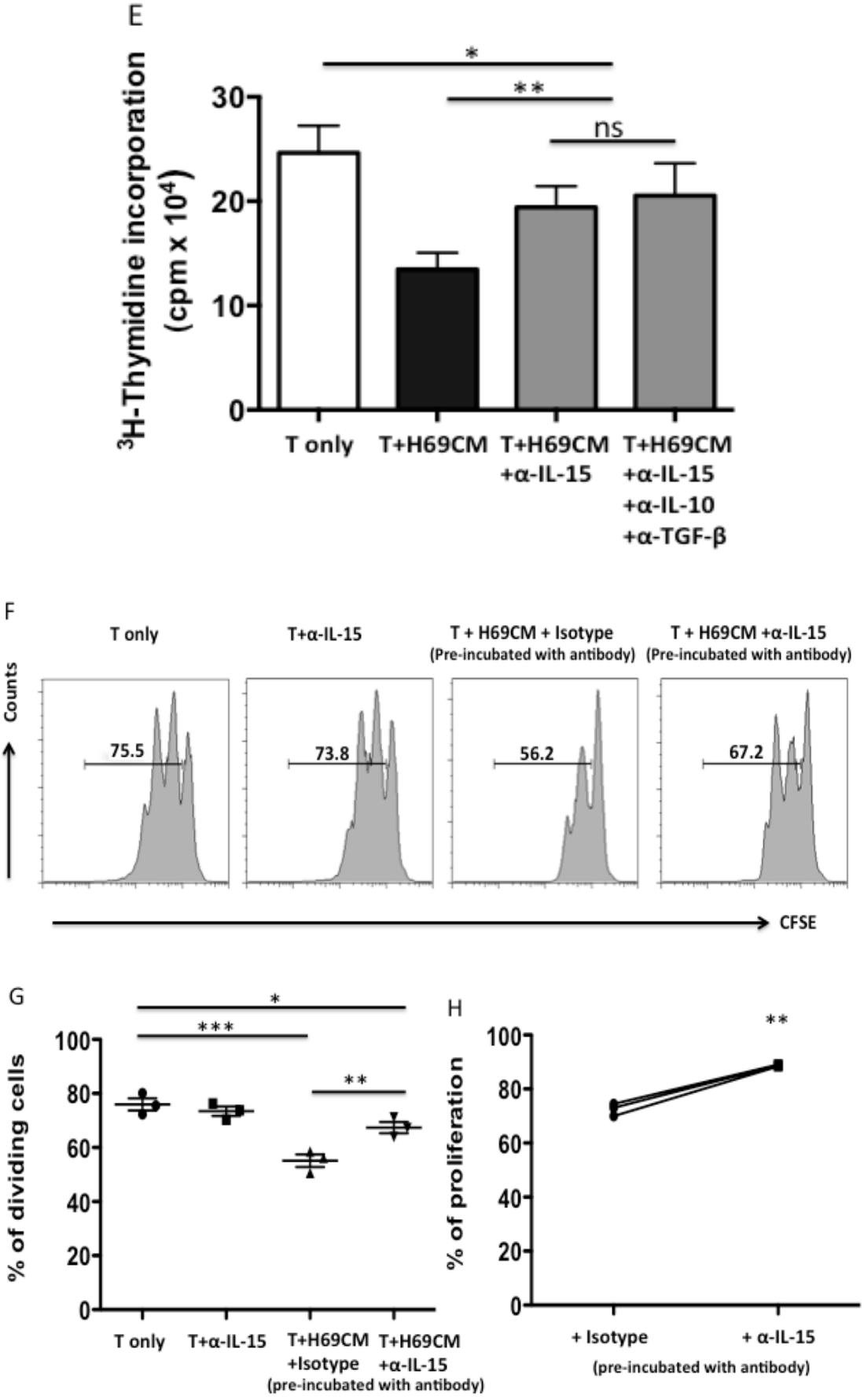
Similarly, the suppression of activated $CD4^+$ T cell proliferation in the presence of H69CM was significantly reversed by addition of IL-15 neutralizing antibody compared to isotype control antibody (Fig. 4.8C). The percentage of $CD4^+$ T cell proliferation was suppressed to 66.2 ± 1.7% by H69CM, but was significantly increased to 83.6 ± 3.1% by neutralization of IL-15 (Fig. 4.8D). The data suggest that blocking of IL-15 activity can partially reverse H69 SCLC cell-induced suppression of $CD4^+$ T cell proliferation. The magnitudes of these effects are similar to the observed above for partial blockade of H69 SCLC cell-induced Treg cell differentiation as a consequence of IL-15 neutralization.

To investigate whether blocking of IL-15, IL-10 and TGF-β activities together can completely abrogate the suppressive effect of H69CM, IL-15 neutralizing antibody, IL-10 receptor blocking antibody and TGF-β receptors inhibitor were added to activated $CD4^+$ T cell culture. There was no difference in H69CM-induced inhibition of $CD4^+$ T cell proliferation between IL-15 neutralizing antibody added alone and all the three antibodies combined together (Fig. 4.8E). The suppressed $CD4^+$ T cell proliferation was significantly reversed in the

presence of IL-15 neutralizing antibody, but a net inhibitory effect remained compared to activated $CD4^+$ T cells cultured alone (Fig. 4.8E).

To ensure the maximum neutralising effect has been achieved, IL-15 neutralizing antibody was next pre-incubated with H69 cells prior to adding H69CM to $CD4^+$ T cell culture. The percentage of proliferating $CD4^+$ T cells in response to activation was significantly reduced by H69CM that was pre-incubated with isotype control antibody (Fig. 4.8F and 4.8G). Pre-incubation of H69 cells in the presence of IL-15 neutralizing antibody significantly increased the percentage of $CD4^+$ T cell proliferation, but remained statistically reduced compared to activated $CD4^+$ T cells cultured alone (Fig. 4.8F, 4.8G and 4.8H). The data suggest that neutralization of IL-15 does not fully abrogate the suppressive effect on $CD4^+$ T cell proliferation that is mediated by soluble factors secreted by H69 SCLC cells. This is consistent with the observed results above that blocking of IL-15 activity can partially reverse H69 SCLC cell-induced suppression of $CD4^+$ T cell proliferation. Taken together the data indicate that IL-15 is an important member of a set of soluble factors produced by H69 SCLC cells to suppress immune responses through induction of functional Treg cells.


A
MLR
^{3}H-Thymidine incorporation (cpm x 10^4)
**
*
0
2
4
6
8
MLR
MLR +α-IL-15
MLR +H69 + Isotype
MLR +H69 + α-IL-15
B
% of proliferation
*
0
20
40
60
80
100
Isotype
+α-IL-15
C
CD4$^+$ T cell proliferation
^{3}H-Thymidine incorporation (cpm x 10^4)
***
**
0
10
20
30
T only
T+α-IL-15
T+H69CM + Isotype
T+H69CM + α-IL-15
D
% of proliferation
**
0
25
50
75
100
Isotype
+α-IL-15


E
*
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ns
30
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^{3}H-Thymidine incorporation (cpm x 10^4)
T only
T+H69CM
T+H69CM +α-IL-15
T+H69CM +α-IL-15 +α-IL-10 +α-TGF-β
F
T only
T+α-IL-15
T + H69CM + Isotype (Pre-incubated with antibody)
T + H69CM +α-IL-15 (Pre-incubated with antibody)
Counts
75.5
73.8
56.2
67.2
CFSE
G
*
***
**
100
80
60
40
20
0
% of dividing cells
T only
T+α-IL-15
T+H69CM +Isotype (pre-incubated with antibody)
T+H69CM +α-IL-15
H
**
100
80
60
40
20
0
% of proliferation
+ Isotype
+ α-IL-15
(pre-incubated with antibody)

Figure 4.8: Blocking of IL-15 activity partially reverses H69 SCLC cell-induced inhibition of MLR and $CD4^+$ T cell proliferation. The experiments were performed as described in Chapter 2. (A) ^{3}H-thymidine uptake by MLR alone or with α-IL-15 antibody or isotype control in the absence and presence of H69 SCLC cells (mean ± SEM, n=5 experiments). * Indicates $p < 0.05$ and ** $p < 0.01$. (B) The percentages of MLR proliferation when co-cultured with H69 cells plus α-IL-15 antibody or isotype control relative to MLR cultured alone. Each line represents an individual donor. (C) ^{3}H-thymidine uptake by $CD4^+$ T cells alone or with α-IL-15 antibody or isotype control in the absence and presence of H69CM (mean ± SEM, n=8 experiments). ** Indicates $p < 0.01$ and *** $p < 0.0001$. (D) The percentages of $CD4^+$ T cell proliferation when cultured with H69CM plus α-IL-15 antibody or isotype control relative $CD4^+$ T cells cultured alone. Each line represents an individual donor. (E) ^{3}H-thymidine uptake by $CD4^+$ T cells alone, or with H69CM, or with H69CM in the presence of α-IL-15 or in combination of α-IL-15, α-IL-10 and α-TGF-β (mean ± SEM, n=3 experiments). * Indicates $p < 0.05$ and ** $p < 0.01$. (F) Representative flow cytometry showing the total populations of dividing $CD4^+$ T cells alone or in the absence and presence of H69CM pre-incubated with α-IL-15 or isotype control. (G) The total populations of dividing $CD4^+$ T cells (mean ± SEM, n=3 experiments). * Indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$. (H) The percentages of $CD4^+$ T cell proliferation when cultured with H69CM plus α-IL-15 antibody or isotyoe control relative to $CD4^+$ T cells cultured alone. Each line represents an individual donor.

4.3 Discussion

The data presented in this chapter demonstrate that H69 SCLC cells produce IL-15 to induce a functional Treg cell population and thereby suppress effector $CD4^+$ T cell proliferation in response to TcR and co-stimulatory molecule activation. This may represent an important mechanism by which SCLC cells suppress immune responses.

The increased population of Treg cells have a phenotype of $CD4^+CD25^+CD127^{low}Helios^-FoxP3^+$. These cells are likely adaptively induced Treg cells as treatment of naïve $CD4^+$ T cells with H69CM recapitulated the population similarly to the effect of the same treatment upon all $CD4^+$ T cells. Currently, the functional study of human Treg cell-mediated suppressive activity is limited to *in vitro* co-culture assays, most of which measure thymidine uptake by cultured T cells in the presence or absence of Tregs. In this chapter, a novel Treg functional assay is established by co-culture of CFSE-labelled naive $CD4^+$ T cells with unlabelled autologous $CD4^+$ T cells previously co-cultured with H69 SCLC cells in the presence of CD3/CD28 stimulation. The induced $FoxP3^+$ cells demonstrate low levels of proliferation but are functional in suppressing the percentage and number of proliferating effector $CD4^+$ T cells. The results are highly consistent with other data from studies of mouse and human Treg cells. These are consistently hypoproliferative *in vitro* but can effectively inhibit polyclonal T cell activation and proliferation responses to stimulation (237, 238).

The precise molecular mechanisms by which regulatory T cells suppress $CD4^+$ T cell proliferation in humans remain to be determined, although they have been extensively studied in mouse and cell models (211). Both human and murine Treg cells may suppress effector cells through physical elimination by direct cell-to-cell contact, the production of suppressive cytokines and the consumption of cytokines and growth factors (239). However, some of the suppressive mechanisms employed by murine Treg cells have not been described in humans (240). Furthermore, there is no direct evidence showing that suppressive mechanisms identified *in vitro* directly reflect *in vivo* suppressive capacities of Treg cells (211). Human Treg cells must be activated through their TcR to be functionally suppressive (241, 242). Once activated, Tregs can exert suppressive effects on a variety of immune cells including effector $CD4^+$, $CD8^+$ T cells, B cells, NK cells, NKT cells, monocytes, macrophages and dendritic cells (DCs). These may be mediated by cell contact and/or soluble factors (211).

Treg cells induced by co-culture of $CD4^+$ T cells with H69 SCLC cells directly suppress effector $CD4^+$ T cell proliferation. The result supports the previous findings that human Treg cells can be suppressive *in vitro* even in the absence of APCs (243, 244). IL-2 consumption by Treg cells can reduce T cell proliferation and induce apoptosis of effector $CD4^+$ T cells (245, 246). The data in Chapter 3 demonstrate that the $CD4^+$ T cell proliferation suppressed by co-culture with H69 cells is not associated with reduction in IL-2 secretion. It may suggest that IL-2 is not required for SCLC cell-induced Treg cell suppressive function, which has been reported by other studies (247, 248).

Induced Treg (iTreg) cells have to date be considered to mediate their suppressive activity through the effects of secreting the immunosuppressive cytokines TGF-β and IL-10 (214). The data here indicate that SCLC cell-induced FoxP3$^+$ cells are likely adaptively induced iTreg cells (Fig. 4.2). However, surprisingly, the suppression of CD4$^+$ T cell proliferation by these iTreg cells is not mediated through IL-10 or TGF-β (Fig. 4.3). Although unlikely, the anti-IL-10 receptor blocking antibody or anti- TGF-β neutralizing antibody/TGF-β signalling inhibitor may not actually be working, future experiments for positive controls need to be performed to exclude this possibility. Previous studies have demonstrated that Treg cells can produce membrane-bound and soluble TGF-β, and that suppression of T cell proliferation can be partially abrogated by blocking TGF-β (249, 250). However, no active TGF-β is detected in the co-culture of H69 SCLC cells with activated CD4$^+$ T cells, and blocking TGF-β fails to reverse the suppression of CD4$^+$ T cell proliferation in response to stimulation. The data suggest the suppressive effect of SCLC cell-induced Tregs is independent of TGF-β, which is similar to other groups' results demonstrating that TGF-β is not involved in Treg-mediated suppression (251, 252). Similarly, whilst IL-10 secretion from activated CD4$^+$ T cells is significantly increased by co-culture with H69 SCLC cells, blocking IL-10 activity fails to abrogate the suppressive effect of induced Treg cells. Therefore, the effect of IL-10 in the SCLC tumour microenvironment may be involved in Treg cell induction and maintenance rather than direct suppressive effect on T cells. This is supported by recent studies demonstrating that IL-10 indirectly influences suppression of CD4$^+$ T cell proliferation by acting on Tregs to maintain FoxP3 expression and suppressive function (253). SCLC cell-induced FoxP3$^+$ cells may therefore

represent a novel subset of iTreg cells. These may be different from iTreg cells that have been previously described and use other mechanisms to suppress $CD4^+$ T cell proliferation (239).

IL-15 is a member of the family of IL-2 cytokines with a molecular weight of ~14kDa. The IL-15 receptor is a heterotrimeric receptor composed of three subunits, (IL-15R) α and β, and the common cytokine receptor γ-chain (γc) (254). IL-15Rα is specific for the IL-15 receptor whilst IL-15Rβ (CD122) is shared with the receptor for IL-2, and γc (CD132) is shared with receptors for IL-2, IL-7, IL-4, IL-9 and IL-21. IL-15 is constitutively expressed by a large number of cells including monocytes, macrophages, dendritic cells, lymphocytes, fibroblasts, epithelial and stromal cells (254). The expression of IL-15 and the number of $IL\text{-}15^+$ cells in healthy normal lung tissue are limited, but increase significantly in inflammatory pulmonary diseases (255). The data in this chapter demonstrate that H69 SCLC cells but not H510 produce IL-15, and malignant cells in tumour biopsies from SCLC patients stain strongly positive for IL-15. The data are consistent with an early study demonstrating that some but not all SCLC cell lines display IL-15 gene expression (256). H69 cell growth is not affected by addition of either recombinant IL-15 protein or blocking IL-15 activity with a neutralizing antibody.

The data demonstrate that IL-15 induces an increased population of functional $FoxP3^+$ Treg cells, and these are responsible for the suppression of the $CD4^+$ T cell proliferative response to activation. The data suggest that IL-15 may have these effects by promoting iTreg cell population to suppress immune response in SCLC. This is consistent with other reports showing that IL-15 can induce

population of FoxP3$^+$ regulatory T cells (235, 257-260). Similar to the data presented here, IL-15 has been shown to increase CD25 and FoxP3 expression on CD4$^+$ T cells to induce Treg cells (235). Furthermore, the presence of IL-15 is required for Treg cell expansion and genetic depletion or neutralization of IL-15 reduces Treg cell population (257-260). Interestingly, neutralization of IL-15 partially blocks induction of Treg cells and IL-10 secretion, and abrogates the suppressed MLR and CD4$^+$ T cell proliferation mediated by H69 SCLC cells. The lack of complete reversal of IL-15-mediated effects is not due to insufficient dose or incubation time of the neutralizing antibody. In the experiments described in this chapter, IL-15 neutralizing antibody was added at high molar excess (200ng/ml), sufficient to neutralize a far higher IL-15 concentration (1ng/ml) than detected in H69 conditioned culture medium. In addition, pre-incubation of IL-15 neutralizing antibody with H69 culture supernatant shows a similar suppressive effect on CD4$^+$ T cell proliferation as the simultaneous incubation. The data suggest that IL-15 is one, but not the only, soluble molecule produced by H69 cells to mediate immune suppression by inducing increased population of Treg cells. Analysis of microarray data shows that in addition to IL-15, IL-1α, IL-11, IL-16, BMP-7 and CSF-2 genes are up-regulated in H69 cells relative to H510 cells. Future experiments may therefore usefully investigate the effect of these cytokines on Treg cell induction.

Chapter 5: Small Cell Lung Cancer Cells Produce Bombesin-like Peptides (BLPs) to Regulate the Phenotype and Function of Monocytes and Monocyte-Derived Macrophages (MDMs)

Chapter 5 Small Cell Lung Cancer Cells Produce Bombesin-like Peptides (BLPs) to Regulate the Phenotype and Function of Monocytes and Monocyte-Derived Macrophages (MDMs).

5.1 Introduction

Bombesin-like peptides (BLPs) are neuropeptides that include bombesin, gastrin-releasing peptide (GRP) and neuromedin (NMB) (Table 5.1) (56). Bombesin and GRP share a conserved bioactive C-terminal heptapeptide sequence that underlies their similar specificities for GRP receptor (GRP-R) and similar physiological effects (57). BLPs have been shown to have mitogenic activity in a large variety of human cancers (261). SCLC cells produce BLPs and GRP that function as autocrine and paracrine growth factors to promote tumour growth and invasion (103). It is well established that many SCLC cells produce both bombesin and GRP at significant concentrations, and GRP mRNA has been detected in H69, H345 and H510 SCLC cell lines (262, 263).

Table 5.1: Bombesin-like peptides (BLPs)

Peptide	Size	Sequence
BN	14-amino-acid	Glu-Gln-Arg-Leu-**Gly-Asn**-Gln-**Trp-Ala-Val-Gly-His-Leu-Met**-NH2
GRP	27-amino-acid	Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-Tyr-Pro-Arg-**Gly-Asn**-His-**Trp-Ala-Val-Gly-His-Leu-Met**-NH2
NMB	10-amino-acid	**Gly-Asn**-Leu-**Trp-Ala**-Thr-**Gly-His**-Phe-**Met**-NH2

There are three subtypes of BLP receptors expressed in mammalian tissues: GRP receptor (GRP-R), NMB receptor (NMB-R) and bombesin receptor subtype 3 (BB3) (56). GRP-R has high affinity for bombesin and GRP and very low affinity for NMB. GRP-R is expressed by the majority of SCLC cell lines characterized to date and is much more commonly found in human malignancies than in normal human tissues (264-266). The production of GRP together with overexpression of GRP-R by tumour cells results in autocrine

growth stimulation and correlates with poor prognosis (267). Moreover, blocking BLPs or GRP-R with neutralizing antibodies or receptor antagonists significantly inhibits SCLC tumour growth both *in vivo* and *in vitro* (64, 268).

BLPs have been shown to affect immune cell function modulating lymphocyte proliferation and antigen presenting cell capability. Early studies have demonstrated that GRP and bombesin can suppress the proliferation of murine lymphocytes in response to activation (269, 270). Lung cancer-derived BLPs including GRP have been shown to inhibit human dendritic cell (DC) maturation and inhibit IL-12 production by DC and T cell activation (271). Furthermore, these neuropeptides are chemoattractants for human monocytes and macrophages and can induce macrophage phagocytosis and activation (272-274).

Macrophages play an indispensable role in the immune system with decisive functions in both innate and adaptive immune response. However, their presence within the tumour microenvironment has been associated with enhanced tumour initiation and progression, promoting cancer cell growth and metastasis, angiogenesis and immunosuppression (145). Two different classes of macrophages (denoted M1 and M2) have been described. These can exert diverse functions depending on the different origins, local environment and responses to challenges (143, 275). Macrophage types *in vivo* and in disease are likely phenotypically more complex than simple M1/M2 types (276). Nevertheless, these models may aid useful discrimination between different types of macrophages behaviour (275). Classically activated (M1) macrophages

are characterized by high antigen-presenting capacity, pro-inflammatory cytokine secretion and production of nitric oxide (NO). They are potent effector immune cells that are capable of killing pathogens and tumour cells (143). Alternatively activated (M2) macrophages are characterized by high phagocytosis capacity and anti-inflammatory cytokine production. They are associated with angiogenesis, wound healing, tissue repair and immunosuppression (143, 275).

In cancer, tumour-associated macrophages (TAMs) appear to play permissive role in disease. Activated macrophages play roles in early tumour pathogenesis, releasing high levels of pro-inflammatory cytokines to sustain the chronic inflammation that seems to be causal in tumour initiation and promotion (277). M1 macrophages secrete TNF-α, IL-6 and IL-1 that are the most common pro-inflammatory cytokines in the tumour microenvironment, therefore, these TAMs may display M1-like phenotype. Once tumours are established, these TAMs may change from an immunologically active state (M1-like) to adopt a protumoral, immunosuppressive (M2-like) phenotype (145). TAMs isolated from established tumours generally display an M2-like phenotype. These TAMs have an IL-2^{low}IL-10^{hi} phenotype, show less antigen presenting and tumoricidal capacity, enhanced phagocytic capacity and high expression of angiogenic factors (278, 279). In contrast to M1 macrophages, TAMs produce less nitric oxide and reactive oxygen intermediates, and express low levels of inflammatory cytokines, and thus may represent a skewed M2 population (280, 281). It has been shown that in NSCLC, macrophages polarize to an M2-like

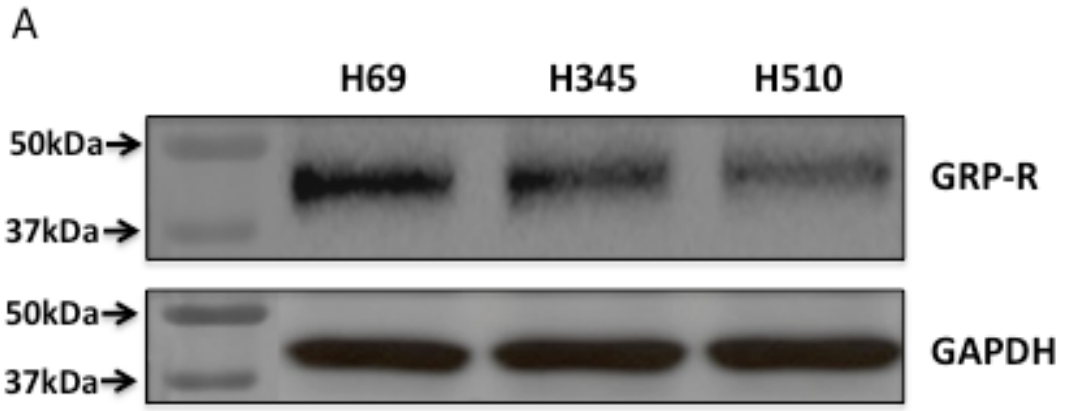
protumoral phenotype during tumour formation, with suppressive effects on T cell responses (282, 283).

I hypothesised that the observed suppressive effects on $CD4^+$ T cells and MLR could be mediated by SCLC cell-derived BLPs. To assess this, I characterised the expression of GRP-R on SCLC cell lines and tumour biopsies, and the effect of blocking BLP signalling on tumour cell growth. I also wished to study whether BLP signalling could affect polarization of monocyte-derived macrophages (MDMs). To this end, *in vitro* co-cultures of H69 cells with MLR and $CD4^+$ T cells in the absence or presence of a GRP-R antagonist were established respectively. The aim was to investigate whether SCLC cell-derived BLP could supress proliferation of MLR and $CD4^+$ T cells. In addition, *in vitro* co-cultures of H69 cells with monocytes and MDMs in the absence or presence of a GRP-R antagonist were established respectively. The aim was to explore the hypothesis that SCLC cell-derived BLPs may regulate the phenotype and functions of monocytes and MDMs.

5.2 Results

5.2.1 SCLC cells and tumour biopsies express gastrin-releasing peptide receptor (GRP-R).

SCLC cells secrete various neuroendocrine growth factors including BLPs and GRP, and their receptors are also widely expressed by SCLC cell lines leading to the development of autocrine and paracrine growth stimulatory loops (101, 102). To investigate whether H69, H345 and H510 SCLC cells express GRP receptor (GRP-R), cell lysates were probed for GRP-R by western blot analysis of SDS-PAGE. GRP-R was expressed in all three SCLC cell lines at different levels with highest expression in H69 cells, intermediate expression in H345 cells and lowest expression in H510 cells (Fig. 5.1A). Immunohistochemistry staining showed that GRP-R was expressed on primary tumour biopsies from SCLC patients (Fig. 5.1B).



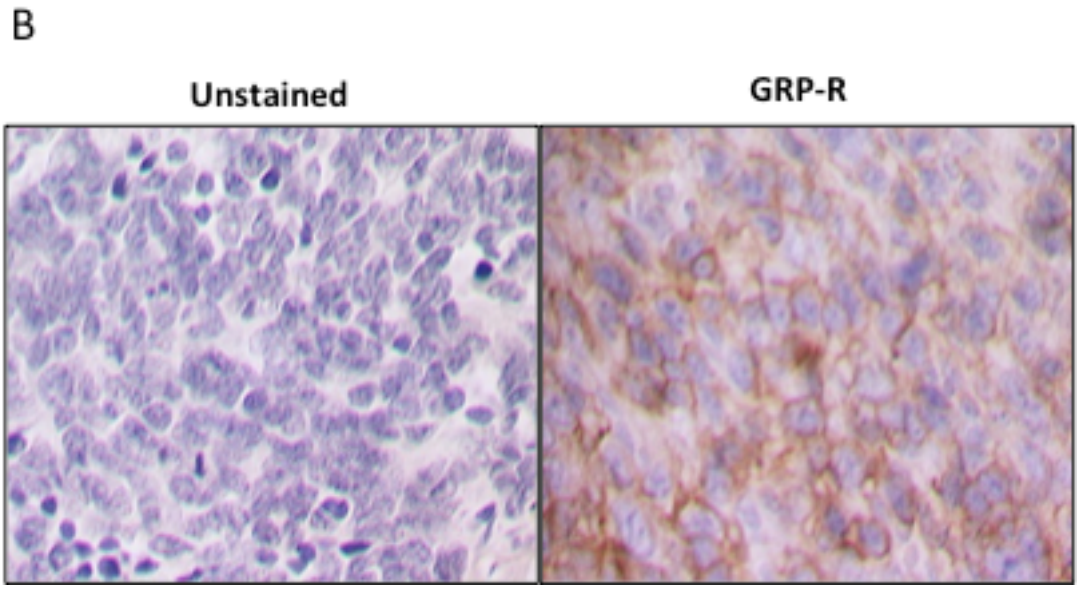


Figure 5.1: SCLC cells and tumour biopsies express GRP-R. (A) Representative western blot for GRP-R in H69, H345 and H510 SCLC cells (n=4 independent experiment). (B) Representative IHC staining for GRP-R in formalin-fixed, paraffin-embedded SCLC tumour sections. Left panel shows unstained tumour biopsy with the anti-GRP-R antibody Right panel shows positive GRP-R staining in a representative tumour biopsy.

5.2.2 RC-3095, a GRP-R antagonist, inhibits the growth of SCLC cells *in vitro.*

Synthetic human GRP-R antagonists bind with high affinity and block the receptor-activated signal transduction pathways. To investigate whether blocking GRP activity can affect SCLC tumour cell growth *in vitro,* RC-3095 (D-TPI6, LEU13 y(CH2-NH)-LEU14)BOMBESIN (6-14), a potent GRP-R antagonist was added to SCLC cell culture. H69, H345 and H510 cells were cultured alone, or with DMSO (5µl), or with RC-3095 in DMSO at concentrations of 1µM, 5µM and 10µM. Cell viability was assessed by MTT assay at 72 hours. There were

no differences in cell viability between SCLC cells (H69, H345 and H510) cultured alone or with DMSO (Fig. 5.2A). Addition of RC-3095 at the concentration of 1μM did not affect H69, H345 or H510 cell viability compared to the cells cultured alone (Fig. 5.2A). However, culture with RC-3095 at higher concentrations (5μM and 10μM) significantly reduced the viability of all the three SCLC cell lines (Fig. 5.2A). The data suggest that blocking bombesin/GRP signalling can inhibit SCLC cell growth *in vitro*.

The phosphoinositide 3-kinase (PI3K/Akt) signalling pathway has been shown to deliver an anti-apoptotic signal to promote cell survival and its dysregulation is implicated in various human cancers (284). To assess whether this pathway is involved in the suppressive effect of RC-3095 on the cell viability of SCLC cells, cell lysates were probed for phospho-Akt (P-Akt) and Akt. As shown, phosphorylation of Akt in H69, H345 and H510 cells was significantly inhibited by the addition of 5μM and 10μM RC-3095 to the cell culture (Fig. 5.2B). The data correlate well with the observed effects of 5μM and 10μM RC-3095 on SCLC cell viability, supporting the hypothesis that treatment with the higher doses of GRP-R antagonist may inhibit SCLC tumour cell growth *in vitro* via the PI3K/Akt signaling pathway.

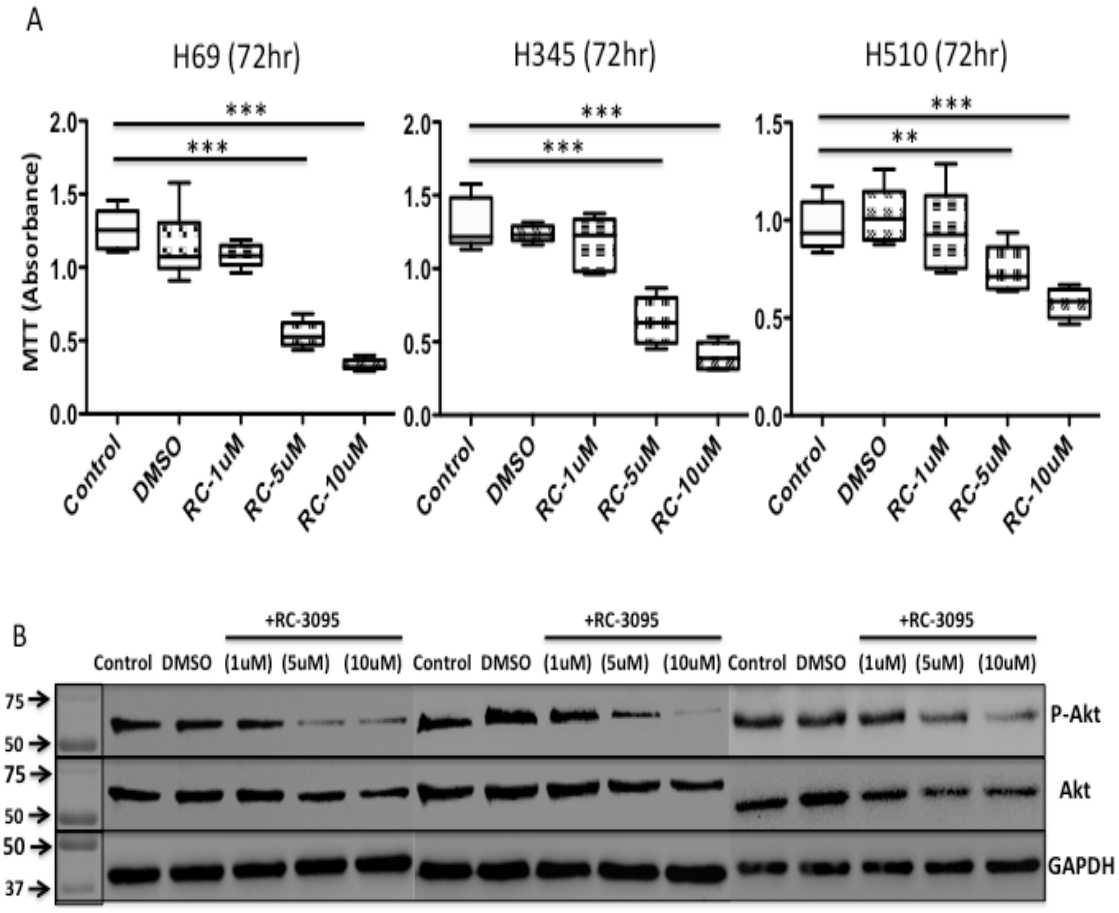


Figure 5.2: RC-3095, a GRP-R antagonist, inhibits the growth of SCLC cells *in vitro*. H69, H345 and H510 cells per well cultured alone, or with DMSO, or with RC-3095 (1μM, 5μM and 10μM) in 24-well plates. Cell viability was assessed by MTT assay at 72 hours. (A) Graph shows the MTT absorbance (mean ± SEM, n=5 experiments). ** Indicates $p < 0.01$ and *** $p < 0.0001$. (B) Representative SDS-PAGE western blot analysis of H69, H345 and H510 cell lysates with different treatments probed for P-Akt, Akt and GAPDH (loading control) (n=3 independent experiment).

5.2.3 Blocking bombesin/GRP signalling has no effect on H69CM-induced suppression of $CD4^+$ T cell proliferation.

BLPs have been shown to suppress proliferation of murine lymphocytes (270). To investigate whether blocking bombesin/GRP signalling could abrogate the

suppression of $CD4^+$ T cell proliferation mediated by H69 conditioned medium (H69CM), RC-3095 was pre-incubated with H69 cells prior to adding H69CM to $CD4^+$ T cell culture. RC-3095 was used at concentration of 1 μM since this concentration has no effect on H69 cell viability (Fig. 5.2). $CD4^+$ T cell proliferation was not affected by addition of RC-3095 compared to the cells cultured alone, but was significantly reduced by H69CM in the presence of DMSO (Fig. 5.3A and 5.3B). However, addition of RC-3095 failed to abrogate the H69CM-suppressed $CD4^+$ T cell proliferation compared to DMSO control (Fig. 5.3A and 5.3B). Blocking of IL-15 activity significantly reversed the suppression of $CD4^+$ T cell proliferation, but combination of RC-3095 had no additional effect on this (Fig. 5.3A and 5.3B). Lack of effect of RC-3095 antagonist on the suppression of T cell proliferation may suggest that bombesin/GRP signalling does not mediate the suppression of $CD4^+$ T cell proliferation caused by factors secreted by H69 cells.

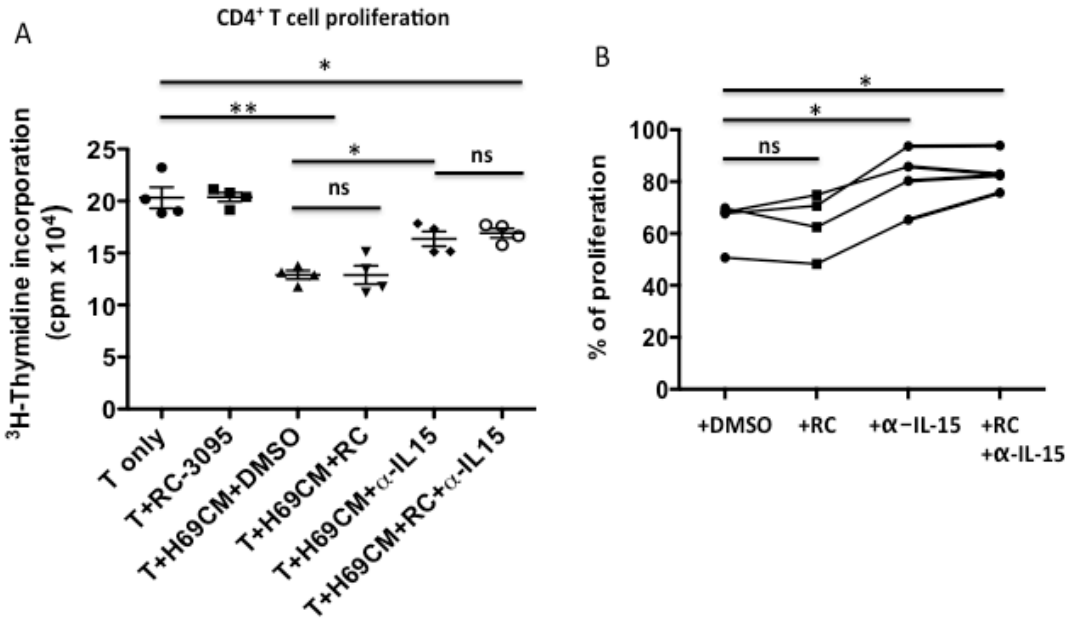


Figure 5.3: Blocking BLP signalling has no effect on H69CM-induced suppression of $CD4^+$ T cell proliferation. (A) ^{3}H-thymidine uptake by $CD4^+$ T cells following activation culture alone or with RC-3095 (1μM), or with 40% H69 CM in the presence of DMSO or RC-3095, or anti-IL-15, or RC-3095 plus anti-IL-15 (mean ± SEM n=4 experiments). * Indicates $p < 0.05$ and ** $p < 0.01$. (B) The percentage of $CD4^+$ T cell proliferation when cultured with H69CM and different additives relative to $CD4^+$ T cells cultured alone or with RC-3095 (n=4 experiments). Each line represents an individual donor. * Indicates $p < 0.05$.

5.2.4 Blocking bombesin/GRP signalling reverses H69 cell-induced suppression of MLR proliferation.

I next wished to investigate whether bombesin/GRP signalling mediates the suppressive effects of H69 cells upon proliferation in the MLR. To this end, 1μM RC-3095 was added to MLR culture system. GRP-R blockade had no significant direct effect upon proliferation in the MLR, whilst co-culture with H69 cells in the presence of DMSO control demonstrated the suppressive effect of this SCLC cell line (Fig. 5.4A and 5.4B). Interestingly, this effect was significantly reversed by the addition of RC-3095. Neutralization of IL-15 also reversed the suppressed MLR proliferation, but remained significantly ($p < 0.05$) reduced compared to MLR cultured alone (Fig. 5.4A and 5.4B). However, the combination of RC-3095 and anti-IL-15 fully abrogated the suppressive effect on MLR mediated by H69 cells (Fig. 5.4A and 5.4B). Together the data suggest that bombesin/GRP signalling, in addition to IL-15 signalling, mediates the suppression of MLR proliferation caused by H69 cells.

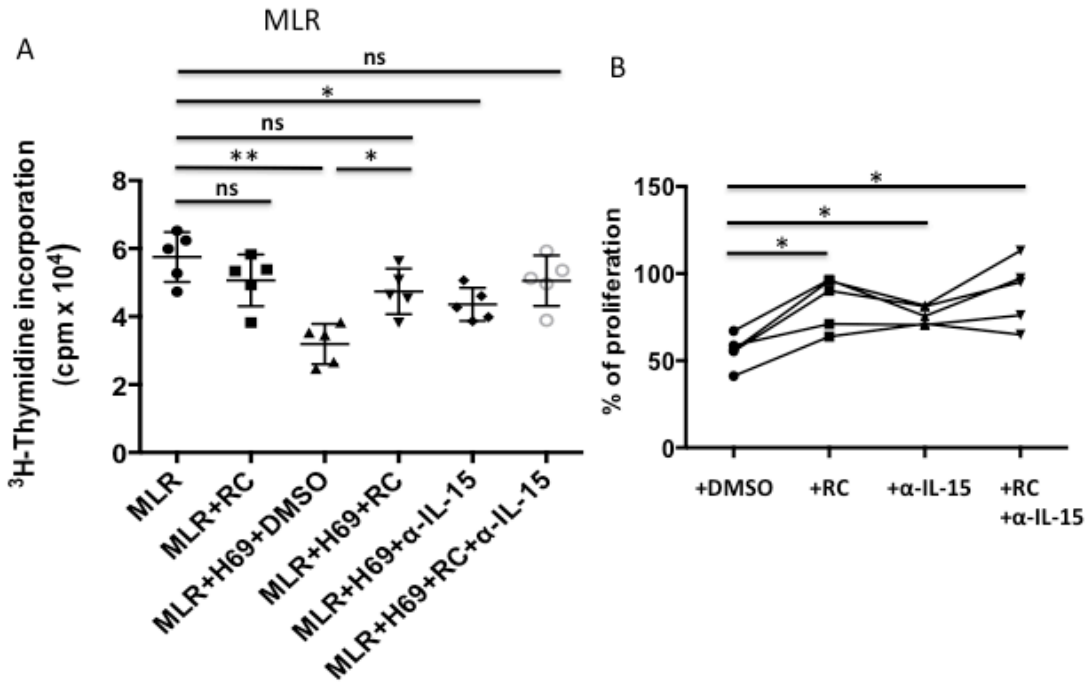


Figure 5.4: Blocking BLP signalling reverses H69-induced suppression of MLR proliferation. (A) Graph represents ^{3}H-thymidine uptake by MLR cultured alone, or with RC-3095 (1μM), or co-cultured with mitomycin C treated H69 cells in the presence of DMSO, or RC-3095, or anti-IL-15, or RC plus anti-IL-15 (mean ± SEM, n=5 experiments). * Indicates $p < 0.05$ and ** $p < 0.01$. (B) Graph shows the percentage of MLR proliferation with different treatments compared to MLR cultured alone or with RC-3095 (n=5 experiments). Each line represents an individual donor. * Indicates $p < 0.05$.

5.2.5 H69 SCLC cells up-regulate GRP-R expression on monocytes and monocyte-derived macrophages (MDMs)

Blocking bombesin/GRP signalling had no effect on H69 SCLC cell-mediated suppression of $CD4^+$ T cell proliferation (Fig. 5.3), but significantly abrogated the inhibition of MLR proliferation (Fig. 5.4). The data suggest that BLPs released by H69 cells might act on other cell types within PBMC population to suppress activation or proliferation of other immune cells. Therefore, I next wished to

determine whether BLPs may have effects on monocytes and monocyte-derived macrophages (MDMs). $CD14^+$ monocytes were isolated and purified to >93% homogeneity, and were differentiated into MDMs displaying a characteristic phenotype of $CD16^+CD68^+$ (Fig. 5.5A). Both monocytes and MDMs expressed GRP-R and its expression was significantly up-regulated by co-culture with H69 SCLC cells (Fig. 5.5B). The data suggest that H69 cell-derived BLPs may interact with monocytes and MDMs through GRP-R.

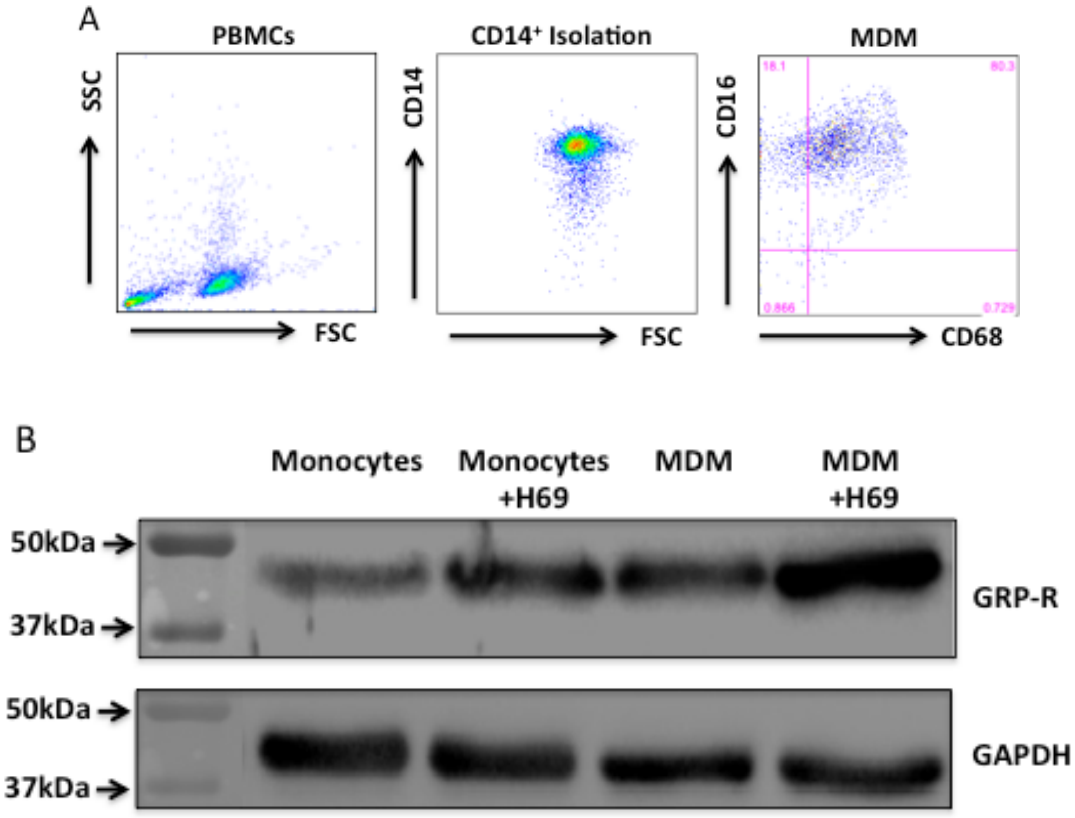


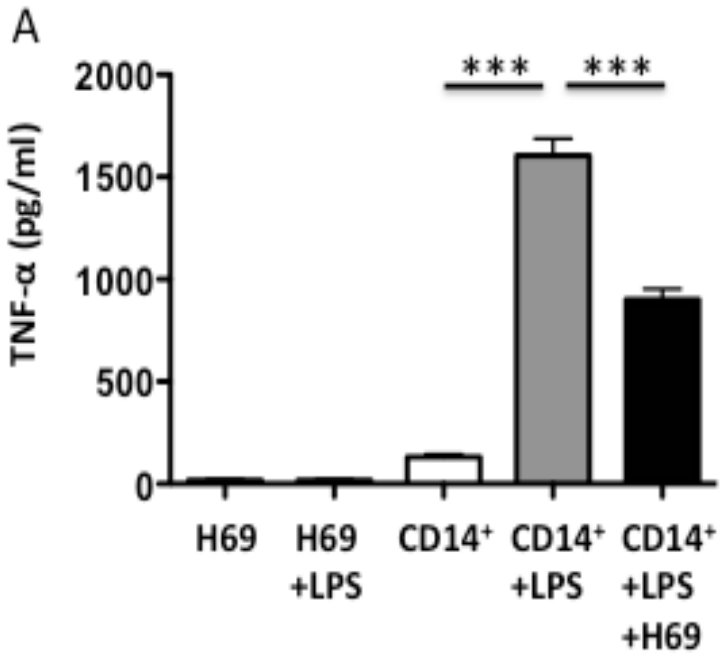
Figure 5.5: H69 SCLC cells up-regulate the expression of GRP-R on monocytes and monocytes-derived macrophages. (A) Representative flow cytometry shows the isolation of PBMCs from peripheral blood, purification of $CD14^+$ monocytes and differentiation of $CD16^+CD68^+$ MDM from monocytes. (B) Representative SDS-PAGE western blot probing for GRP-R in cell lysates (n=3 independent experiment).

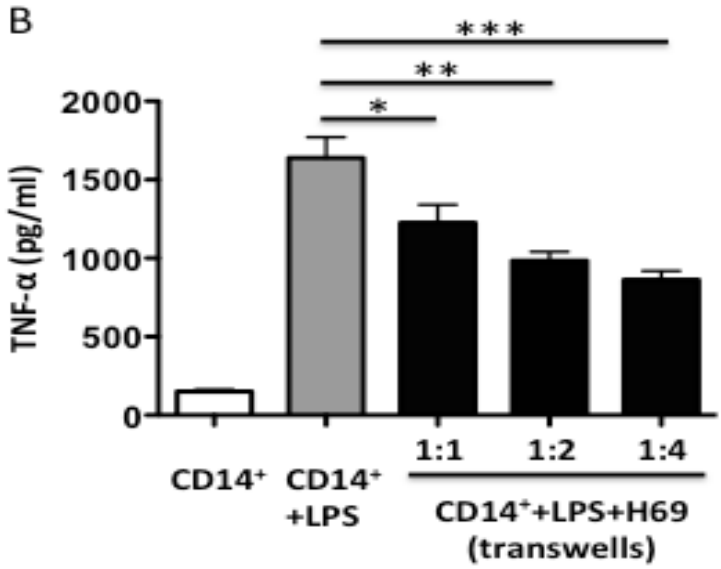
5.2.6 BLPs suppress LPS-induced activation of monocytes.

To investigate whether H69 SCLC cells can inhibit monocyte activation, H69 cells were co-cultured with $CD14^+$ monocytes. LPS was added as a pro-inflammatory stimulus and TNF-α release was measured as a product of monocyte activation. H69 cells cultured alone or with LPS did not produce TNF-α in the supernatant. TNF-α secretion was low when monocytes were cultured alone without stimulation, but was significantly increased in the presence of LPS (Fig. 5.6A). Interestingly, TNF-α production from LPS-stimulated monocytes was significantly reduced by co-culture with H69 cells (Fig. 5.6A). To determine whether the suppressive effect on TNF-α secretion was dependent on formation of cell contacts, transwells were used to separate the cells. TNF-α production from activated monocytes was significantly reduced by co-culture with H69 cells in a dose-dependent fashion (Fig. 5.6B). To investigate whether H69 culture supernatant can also have the inhibitory effect on monocyte activation, H69 conditioned medium (H69CM) or PBS were added. There was no difference in TNF-α secretion between monocytes cultured alone or with addition of PBS. However, it was significantly reduced in the presence of H69 culture supernatant (Fig. 5.6C). Together the data suggest that H69 SCLC cells produce soluble factors that are capable of suppressing TNF-α secretion from LPS-stimulated monocytes.

To determine whether blocking bombesin/GRP signalling could reverse the suppressive effect of H69 cells on TNF-α secretion by LPS-stimulated monocytes, the effects of 1μM RC-3095 were assessed. Addition of DMSO or

RC-3095 did not affect the viability of LPS-stimulated monocytes cultured alone or in co-culture with H69 cells (Fig. 5.6D). TNF-α production was not affected by addition of DMSO or RC-3095 to LPS-stimulated monocytes, but was significantly reduced by co-culture with H69 SCLC cells alone or in the presence of DMSO (Fig. 5.6E). Interestingly, the suppression of TNF-α secretion was significantly reversed by addition of RC-3095, and the suppressive effect was abrogated compared to monocytes cultured alone (Fig. 5.6E). The data suggest that H69-derived BLPs may suppress activation of LPS-stimulated monocytes and that the suppressive effect can be abrogated by blocking bombesin/GRP signalling.

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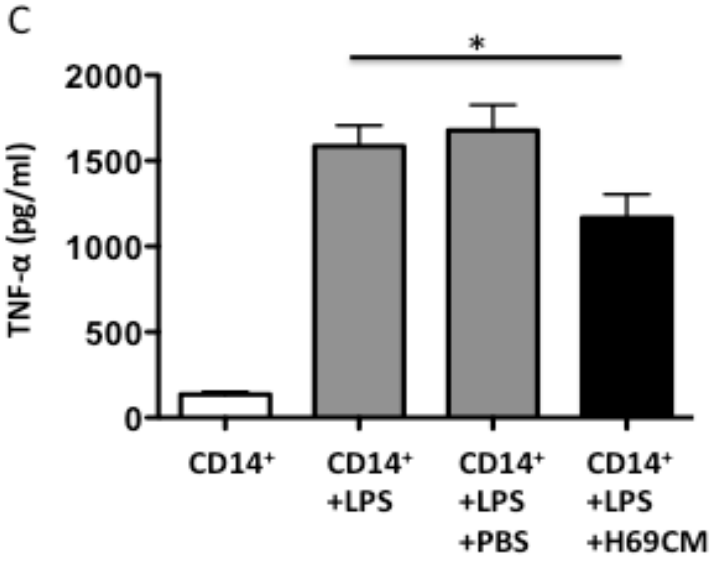
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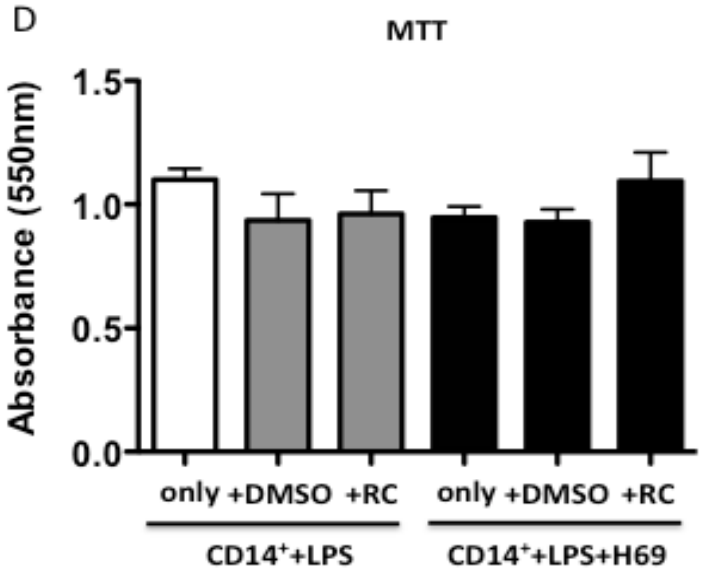
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CD14+ +LPS+H69

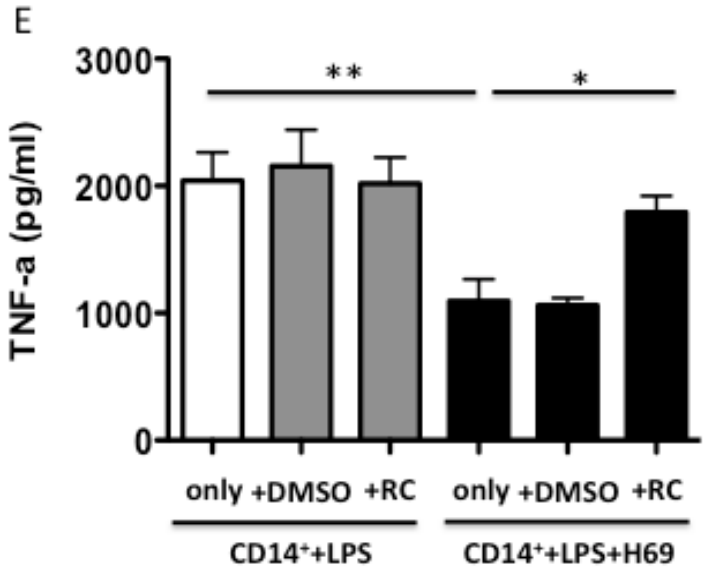


Figure 5.6: BLPs suppress LPS-induced activation of monocytes. The experiments were performed as described in sections 2.6 and 2.12 Chapter 2. (A) TNF-α concentration in culture supernatant from H69 cells cultured alone or with LPS, or monocytes cultured alone or with LPS in the absence or presence of H69 cells assessed by ELISA. (Mean ± SEM, n=4 experiments). *** Indicates $p < 0.0001$. (B) TNF-α concentration in culture supernatant from monocytes cultured alone or with H69 cells in the presence of LPS assessed by ELISA. (Mean ± SEM, n=3 experiments). * Indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$. (C) TNF-α concentration in culture supernatant from monocytes cultured alone or with 40% H69CM or PBS in the presence of LPS (Mean ± SEM, n=4 experiments). * Indicates $p < 0.05$. (D) Graph shows the cell viability by monocytes following activation with LPS, cultured alone or with H69 cells in the presence of DMSO or RC-3095 assessed by MTT assay (Mean ± SEM, n=3 experiments). (E) Graph shows TNF-α concentration (Mean ± SEM, n=3 experiments). * Indicates $p < 0.05$ and ** $p < 0.01$.

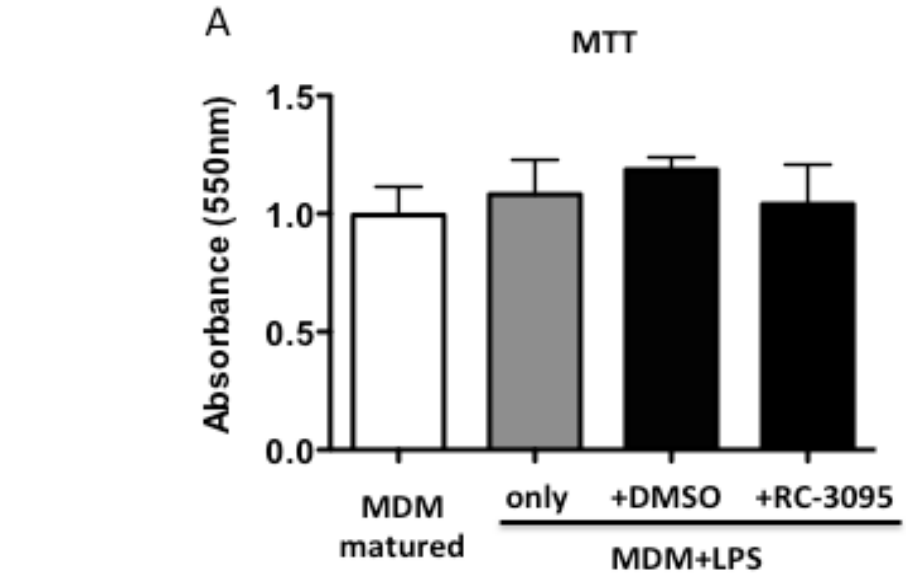
5.2.7 BLPs can induce alternatively activated macrophage phenotype.

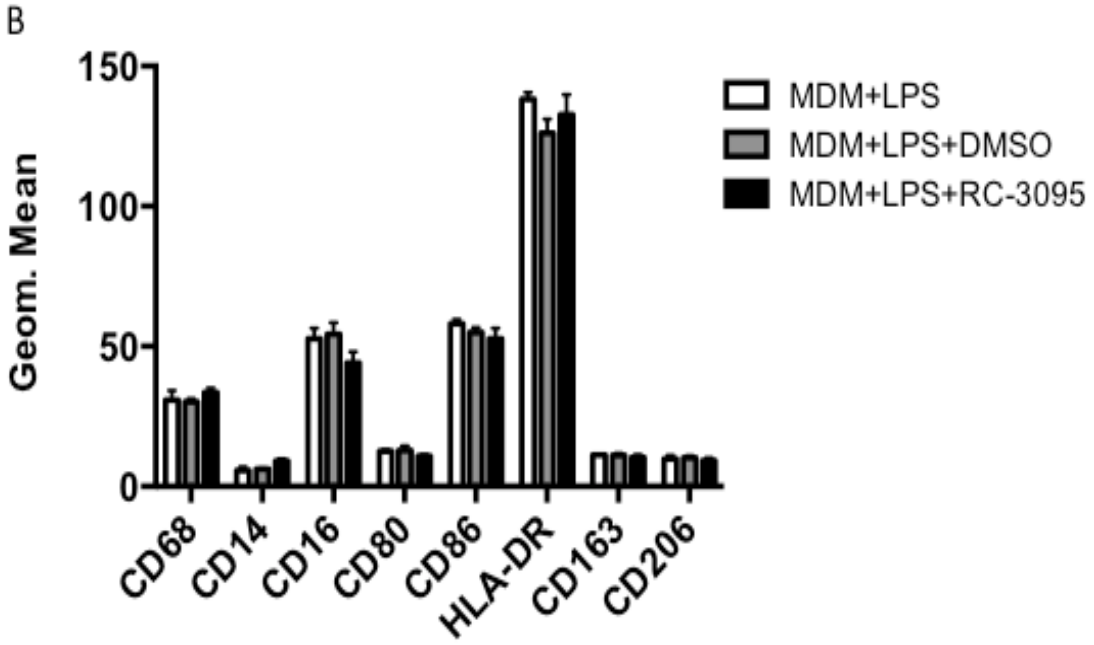
To investigate whether blocking bombesin/GRP signalling could affect cell viability or the phenotypes of activated MDMs, 1μM RC-3095 was added.

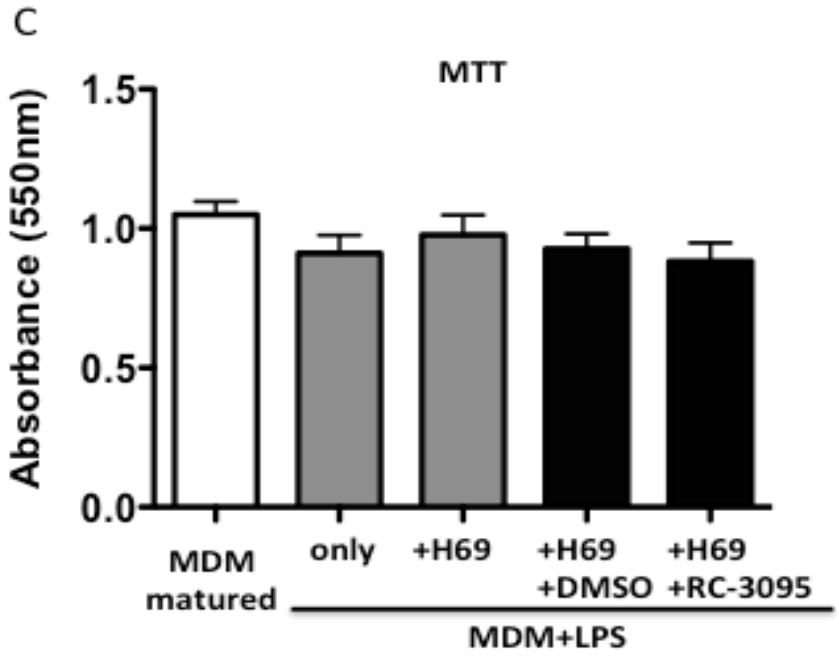
Addition of RC-3095 did not affect cell growth or the expression of various cell markers on LPS-stimulated MDMs (Fig. 5.7A and 5.7B).

Reliable cell-surface markers of the classically activated macrophage (M1) phenotype are not well defined in humans, but M1 macrophages express CD16 and CD86 upon activation. Alternatively activated macrophages (M2) express high levels of CD206 (mannose receptor) and CD163 (scavenger receptor) on the cell surface (285, 286). To determine whether H69 cell-derived BLPs could affect cell viability or macrophage phenotype polarization, MDM were matured, activated with LPS and co-cultured with H69 cells in the presence of RC-3095. Co-culture of MDMs with H69 cells or blocking bombesin/GRP signalling with RC-3095 did not affect the viability of MDMs (Fig. 5.7C). Flow cytometry studies identified no differences in the intracellular expression of CD68, or in the surface expression of CD80 or HLA-DR on LPS-activated MDMs among the different treatments (Fig. 5.7D). Interestingly, co-culture with H69 cells alone or in the presence of DMSO significantly reduced the surface expression of CD16 and CD86 on LPS-activated MDM compared to the MDMs cultured alone (Fig. 5.7E). However, the suppressive effect was completely reversed by addition of RC-3095 (Fig. 5.7E). The data suggest that SCLC cell-derived BLPs has no effect on MDM maturation, but can down-regulate CD16 and CD86 expression on MDMs in response to activation. Furthermore, co-culture with H69 cells alone or in the presence of DMSO significantly up-regulated the surface expression of CD163 and CD206 on LPS-activated MDMs compared to the MDMs cultured alone (Fig. 5.7E). However, addition of RC-3095 completely prevented this increase (Fig. 5.7E). Together the data suggest that H69 SCLC cells may

induce an M2-like (alternatively activated macrophage) phenotype in matured MDMs.



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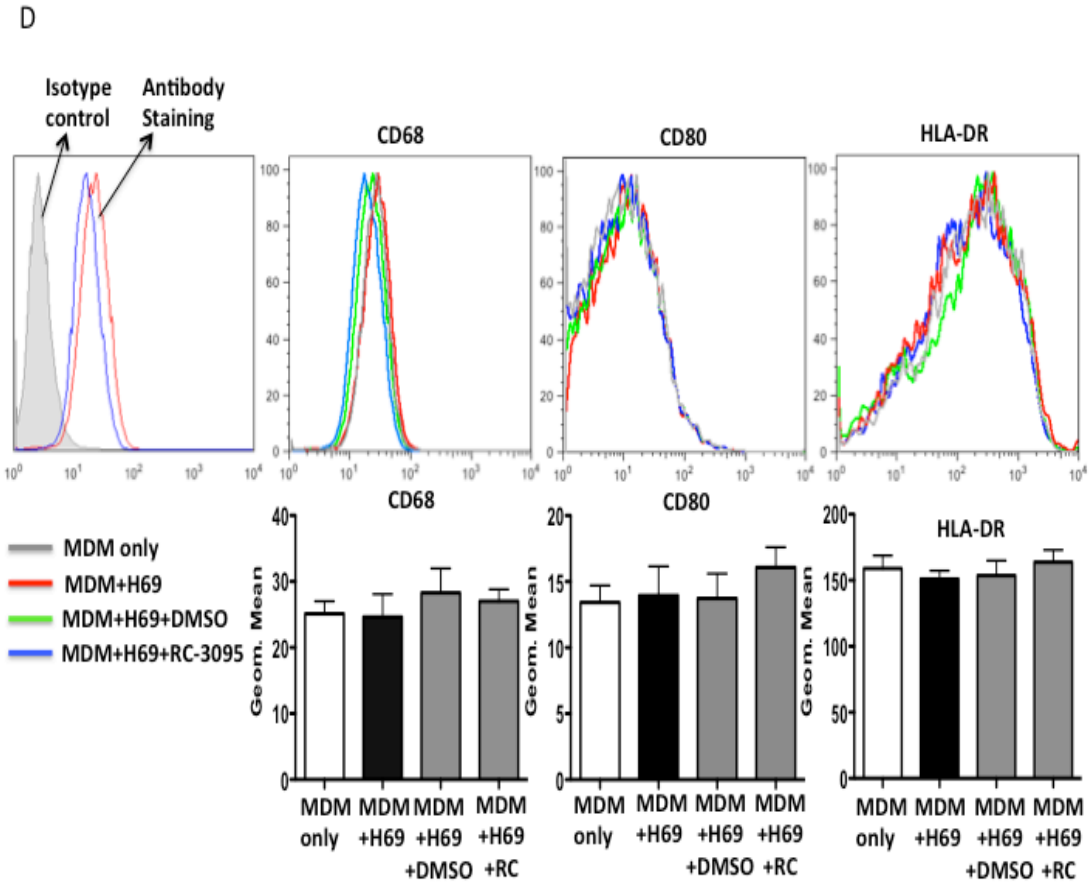
MDM+LPS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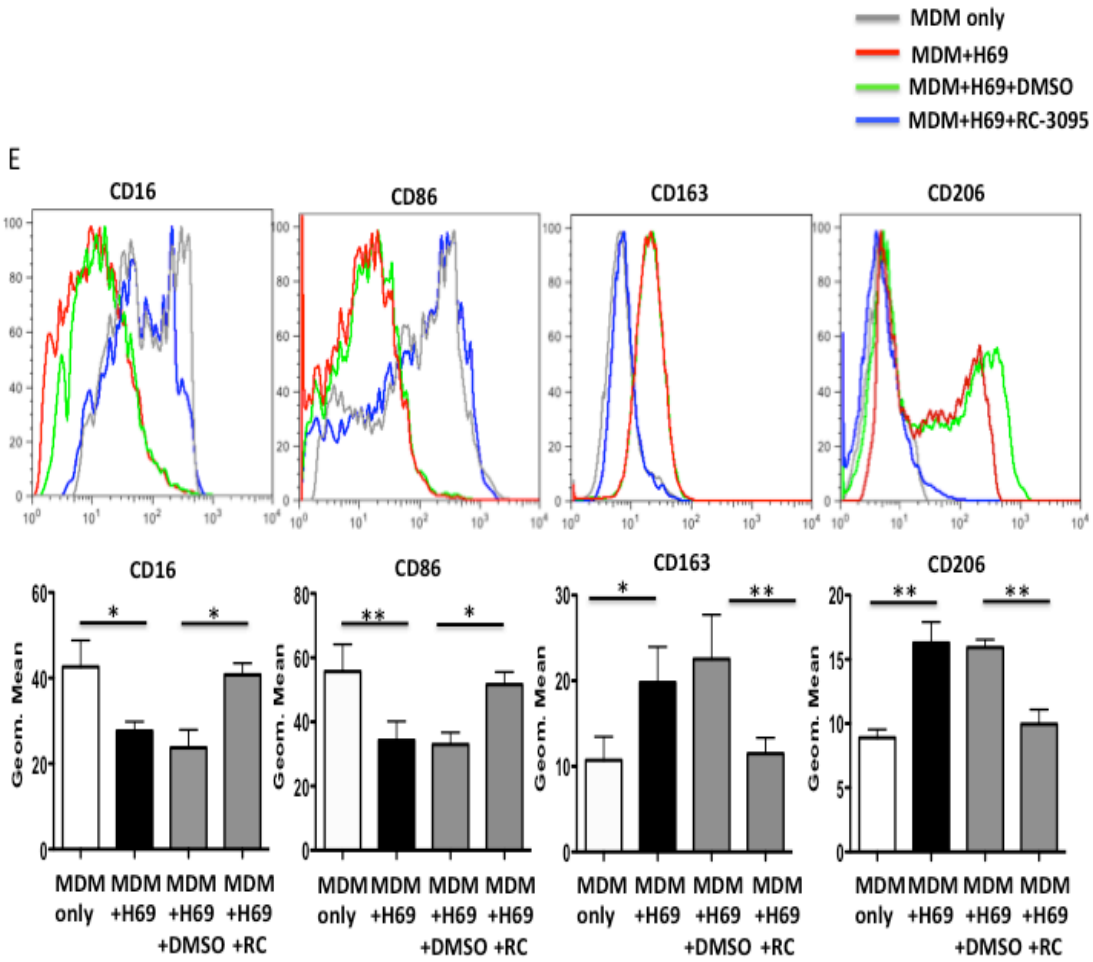


Figure 5.7 BLPs induce alternatively activated macrophage phenotype. The experiments were performed as described in sections 2.6 and 2.12 Chapter 2. (A) Viability of matured MDM cultured alone or with LPS in the presence of DMSO or RC-3095 assessed by MTT assay (Mean ± SEM, n=3 experiments). (B) Expression of CD68, CD14, CD16, CD80, CD86, HLA-DR, CD163 and CD206 on MDM (Geom. Mean ± SEM, n=3 experiments). (C) MDM viability assessed by MTT assay (Mean ± SEM, n=4 experiments). (D) Representative flow cytometry shows the intracellular expression of CD68 and cell surface expression of CD80 and HLA-DR, and bar graph shows the average fluorescence intensity (Geom.Mean ± SEM, n=4 experiments). (E) Representative flow cytometry shows the expression of CD16, CD86, CD163 and CD206, and bar graph shows the average fluorescence intensity (Geom.Mean ± SEM, n=4 experiments). * Indicates $p < 0.05$ and ** $p < 0.01$.

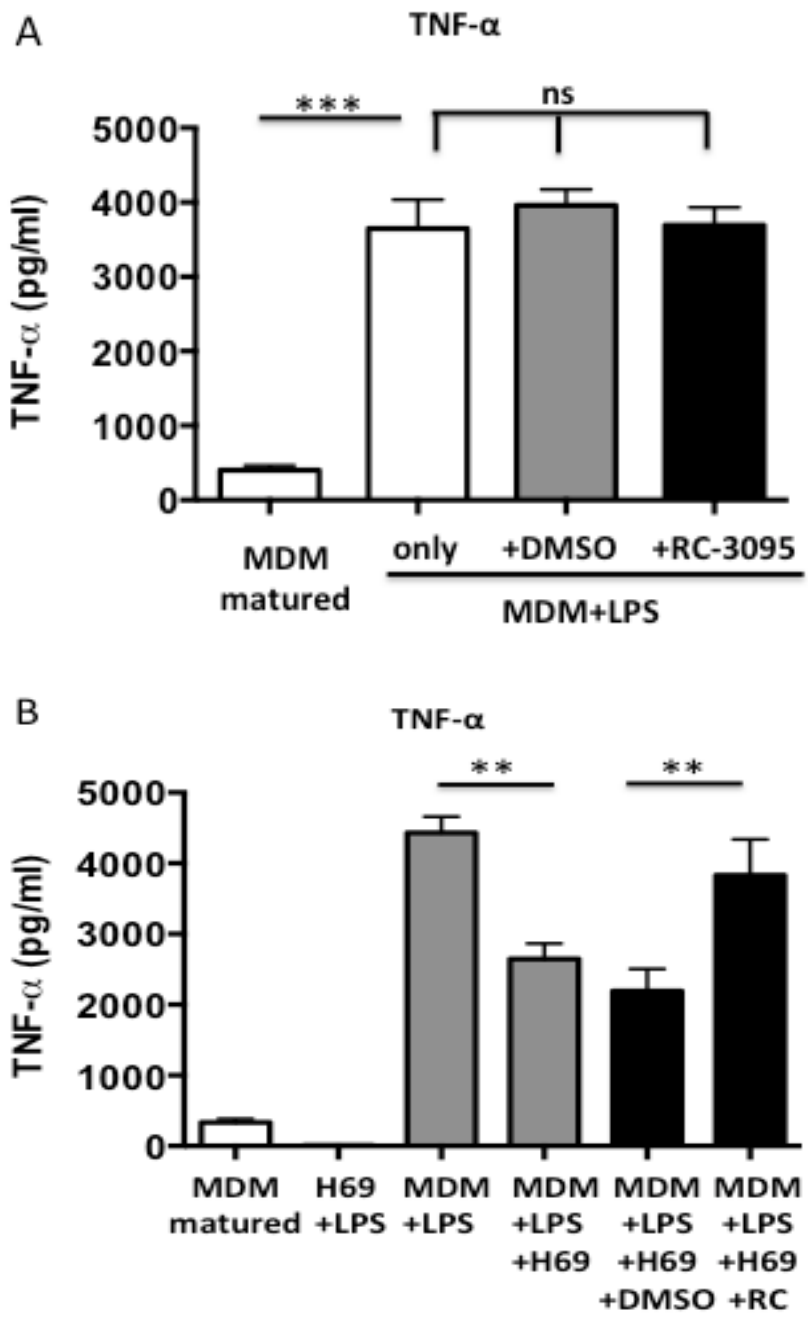
5.2.8 BLPs suppress TNF-α and IL-6 production, and increases IL-10 secretion from activated MDMs.

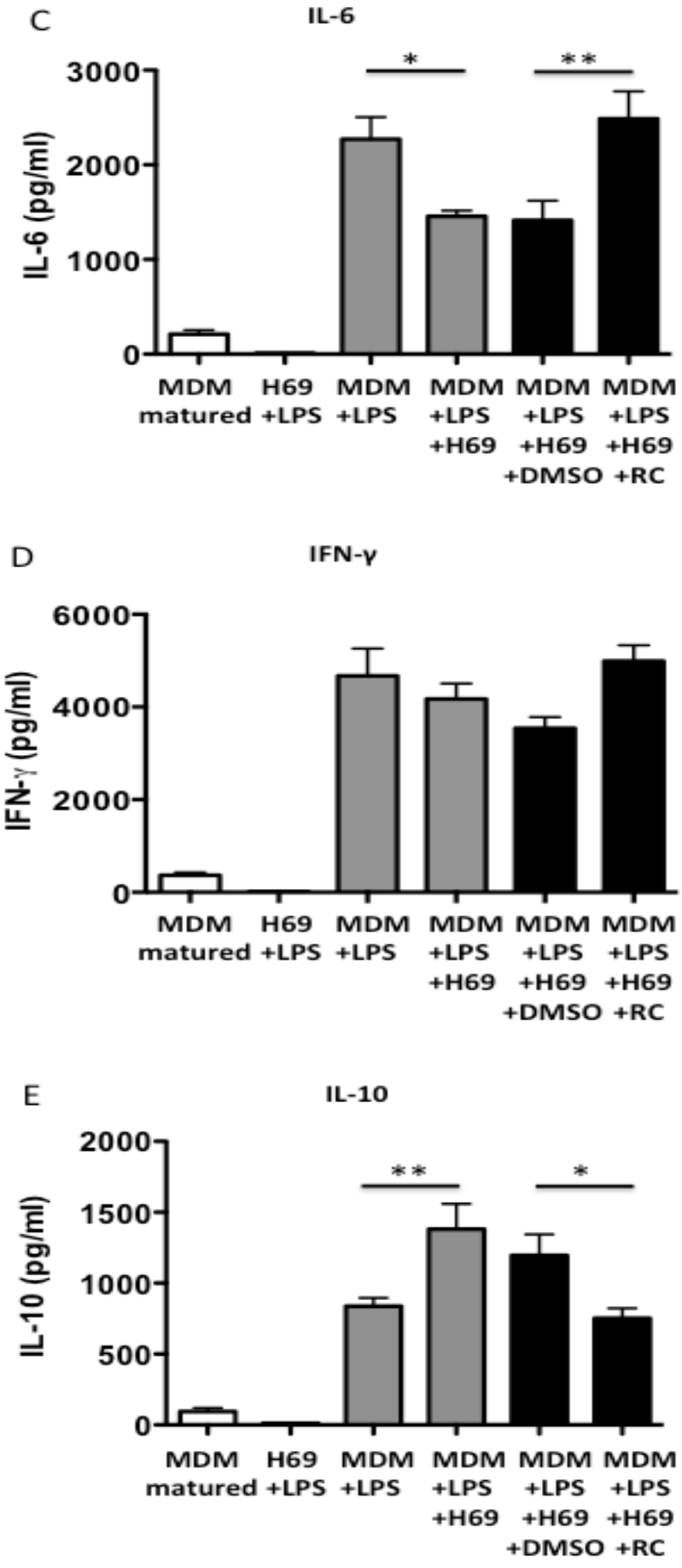
To determine whether blocking bombesin/GRP signalling has an effect on TNF-α secretion from MDMs upon activation, RC-3095 was used. LPS stimulation significantly increased TNF-α secretion, but addition of RC-3095 did not affect TNF-α production from LPS-activated MDMs (Fig. 5.8A).

M1 macrophages are characterized by secretion of pro-inflammatory cytokines such as TNF-α, IL-6, IFN-γ and IL-1, resulting in an effective pathogen killing mechanisms. Conversely, M2 macrophages release large amounts of anti-inflammatory cytokines such as IL-10 or TGF-β, thus may suppress immune responses and contribute to tumour cell growth (278). BLPs produced by H69 cells induced phenotypically M2-like macrophages (Fig. 5.7). To investigate whether the M2-like macrophage induction is associated with alterations in cytokine secretion from MDM, the supernatants from cell culture of activated MDM co-culture with H69 cells in the presence of RC-3095 were collected. TNF-α, IL-6, IFN-γ and IL-10 production was measured by ELISA.

TNF-α and IL-6 secretion by MDMs was significantly increased with LPS stimulation, but was significantly reduced by co-culture with H69 cells (Fig. 5.8B and 5.8C). Addition of RC-3095 significantly reversed this suppressive effect (Fig. 5.8B and 5.8C). There were no significant differences in IFN-γ production from activated MDMs cultured alone or in co-culture with H69 cells in the presence of RC-3095 or DMSO (Fig. 5.8D). IL-10 secretion from activated

MDMs was significantly increased by co-culture with H69 cells, but this increase was abrogated by addition of RC-3095 (Fig. 5.8E). The data demonstrate that H69 cell-derived BLPs can suppress TNF-α and IL-6 pro-inflammatory cytokine secretion and induce anti-inflammatory IL-10 production from MDMs in response to activation.

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IL-6 (pg/ml)
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MDM matured
H69 +LPS
MDM +LPS
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MDM +LPS +H69 +DMSO
MDM +LPS +H69 +RC

Figure 5.8: BLPs suppress TNF-α and IL-6 production, and increases IL-10 secretion from activated MDMs. (A) TNF-α concentration by MDM cultured alone, or activated with LPS or in the presence of DMSO or DMSO or RC-3095 assessed by ELISA (Mean ± SEM, n=3 experiments). *** Indicates $p < 0.0001$. (B-E) TNF-α, IL-6, IFN-γ and IL-10 concentration by matured MDM, or LPS-stimulated MDM cultured alone or with H69 cells in the presence of DMSO or RC-3095 assessed by ELISA respectively (Mean ± SEM, n=3 experiments). * Indicates $p < 0.05$ and ** $p < 0.01$.

5.3 Discussion

The data presented in this chapter demonstrate that H69, H345 and H510 SCLC cells express GRP-R and that blocking bombesin/GRP signalling inhibits SCLC cell growth in concert with reductions in PI3K/Akt signalling. SCLC cell-derived BLPs can induce an M2-like alternatively activated macrophage phenotype from MDM in response to LPS activation. This polarization is associated with reduced secretion of TNF-α and IL-6, and increased production of IL-10 from LPS-activated MDMs. This therefore represents another mechanism by which SCLC cells suppress the immune response.

In this chapter, GRP-R expression is observed in H69, H345 and H510 SCLC cell lines at different levels. The data is consistent with early studies showing that GRP-R mRNA can be detected by RT-PCR in majority of SCLC cell lines including H69 and H345 cells (287, 288). The expression of GRP-R is much more commonly found in various human malignancies than in normal tissues (56). In addition, GRP-R expression in lung tissues has been reported to be significantly elevated with tobacco exposure (289) and cigarette smoke is the most significant risk factor for lung cancers. Therefore, GRP-R expression is often overexpressed in lung cancers (264) and is more frequent in lung cancer patients than cancer-free control subjects (290). The data presented here show that GRP-R is expressed on primary tumour biopsies from SCLC patients. The data are consistent with other studies demonstrating that GRP-R mRNA is detectable in SCLC tumour cells (291) and GRP-R is expressed on tumour biopsies from a large population of patients with SCLC (292). Future

experiments to study whether the expression of GRP-R on tumour biopsies is associated with patient survival in SCLC may now be indicated.

Blocking bombesin/GRP signalling with the GRP-R antagonist RC3095 inhibits H69, H345 and H510 SCLC cell growth *in vitro*. These data are consistent with early studies demonstrating that BLPs are autocrine growth factors in SCLC (103). Similar effects of RC-3095 have been shown to inhibit SCLC cell growth *in vivo*. In animal studies, subcutaneous treatment of nude mice xenografted with human H69 SCLC cells with bombesin significantly increased tumour weight (293). However, blocking bombesin/GRP signaling with RC-3095 significantly reduced the tumour volume by 50-70% (294). Similarly, RC-3095 reduced tumour growth in nude mice xenografted with human H128 SCLC cells (64). In addition, blocking bombesin/GRP activity with analogues or with other receptor antagonists can also inhibit SCLC tumour cell growth *in vivo* and *in vitro* (295-297). The suppressive effect of RC-3095 on SCLC cell proliferation is dose-dependent. Addition of RC-3095 at concentrations of 5 μM and 10 μM suppress SCLC tumour cell growth, whilst 1 μM has no effect. The data are consistent with a previous observation demonstrating that bombesin does not stimulate the proliferation of SCLC cell lines at concentrations between 0.1 nM and 1 μM (298). The reduced cell viability is associated with reduced phosphorylation of Akt in SCLC cells, supporting the hypothesis that the PI3K/Akt signaling pathway may mediate proliferation in SCLC cells.

Neuropeptides play an important role in the regulation of function and survival of immune cells (299). There are no previous data on the interactions between

SCLC-derived BLPs and immune cells. The data in this chapter demonstrate that blocking bombesin/GRP signalling has no effect on the suppressive effect of H69 cells on $CD4^+$ T cell proliferation, but reverses their MLR suppressive effects. Therefore, such BLPs secreted by H69 cells may not be responsible for the direct observed suppression of $CD4^+$ T cell proliferation, but instead act on other immune cells to inhibit their capacity to induce T cell proliferation (271, 300). On the other hand, lack of effect of RC-3095 at the concentration of 1 μM on the suppression of T cell proliferation may be due to insufficient dose, future experiments with a series of concentrations of RC-3095 need to be performed to address this issue.

GRP-R is expressed by monocytes and monocyte-derived macrophages (MDMs), and its expression is significantly up-regulated by co-culture with H69 cells. The data suggest that H69 cell-derived bombesin/GRP could affect monocytes or MDMs through its interaction with GRP-R. Other studies have demonstrated that GRP-R is expressed by immune cells (301) and GRP may modulate monocyte and macrophage activities (272-274). Recent studies have shown that GRP can induce inflammation (302, 303). Blocking GRP activity with RC-3095 has anti-inflammatory effects in arthritis and sepsis models, down-regulating the production of pro-inflammatory cytokines TNF-α, IL- 1β and IL-6 (304). The data in this chapter demonstrate that the secretion of TNF-α from LPS-activated monocytes is suppressed by co-culture with H69 cells or H69 culture supernatant and that this suppression is mediated by bombesin/GRP signaling.

H69 cell-derived BLPs have no effect on MDM survival but can modulate the phenotype and function of MDM upon activation. The expression of CD16 and CD86 is significantly down-regulated on LPS-activated MDMs. CD16, the receptor for the Fc portion of IgG, is important in antibody-mediated cell cytotoxicity and CD86 is an important co-stimulatory molecule for T-cell activation and proliferation (285). M1 macrophages are tumoricidal, function as antigen-presenting cells and express high levels of CD16 and CD86 on the cell surface (285, 305). Therefore, down-regulation of these surface molecules by SCLC cell-derived BLPs may inhibit the anti-tumour function of macrophages and prevent activation of T cells within the tumour microenvironment. It has been shown that in some solid tumours, although abundant macrophages are found, there is little evidence of immune rejection, and the local tumour microenvironment is immunosuppressive with few $CD8^+$ cytotoxic and $CD4^+$ effector T cell infiltration (306). This may be associated with reduced expression of CD16 and CD86 on the surface of antigen presenting cells, which could impair their capacity to activate effective immune responses.

In addition, H69 cell-derived BLPs significantly increase the expression of CD163 and CD206 on MDM upon activation. CD163 (scavenger receptor) and CD206 (mannose receptor) are both highly expressed on M2 macrophages that are characterized by high phagocytic capacity and anti-inflammatory cytokine production (286, 307). Macrophages co-expressing CD163 and CD206 have high capacity for apoptotic cell uptake and produce high levels of IL-10 and CCL18 (308, 309). The data in this chapter suggest that upon activation, rather than being M1 tumoricidal macrophages, MDMs can be induced to M2-like

immunosuppressive phenotype macrophages by SCLC cell-derived BLPs. This may support the recent studies on substance P (SP), another neuropeptide that is associated with SCLC cell growth (310), demonstrating that SP is able to shift macrophages to an M2 activation state and induce CD163 expression (311, 312).

The modulation of macrophage cell surface phenotype mediated by H69 cell-derived BLPs is validated by the findings of consistent alterations in cytokine production. In the presence of GM-CSF and LPS stimulation, macrophages are induced to adopt an M1 phenotype and produce high levels of pro-inflammatory cytokines including TNF-α, IL-6, IL-12 and IL-1β (285, 286). M2 macrophages are characterized by low production of these pro-inflammatory cytokines and high production of the anti-inflammatory cytokine IL-10 (285, 286). The data in this chapter demonstrate that the secretion of TNF-α and IL-6 is significantly reduced and IL-10 production is significantly increased from MDMs activated with LPS through GRP-R signalling. The data therefore indicate that SCLC cell-derived BLPs may induce a switch from M1-like to M2-like macrophage phenotype in terms of both cell surface markers and cytokine production. Polarization of macrophages to an M2-like phenotype by proximity to SCLC cells may therefore represent another mechanism by which SCLC tumours can suppress the immune response.

Chapter 6: Identification of an Independent Prognostic Marker in Small Cell Lung Cancer

Chapter 6: Identification of an Independent Prognostic Marker in Small Cell Lung Cancer

6.1 Introduction

Immune cells in the tumour microenvironment can eradicate malignant cells and control tumour growth. Tumour-infiltrating lymphocytes (TILs), macrophages and dendritic cells (DC) that are commonly found in solid tumours have been associated with improved patient survival and reduced relapse in various cancers (313-317). However, some studies have demonstrated that, although multiple immune cell types commonly infiltrate solid tumours, they may fail to demonstrate effective, consistent and durable anti-tumour responses (318). One of the most important reasons may be that $FoxP3^+$ Treg cells within the tumour microenvironment play a significant role in suppressing anti-tumour immune responses (176).

The published data on the impact of Treg infiltration in the tumour microenvironment on patient survival is, however, controversial. The presence of higher proportions of $CD4^+FoxP3^+$ Treg cells among TILs has been associated with poor survival in melanoma, ovarian, breast and gastric cancers (224, 319, 320). In contrast, other reports demonstrate that large numbers of Treg cell infiltration is associated with improved survival in Hodgkin lymphoma, head/neck, urinary bladder and colon cancers (321-324). Furthermore, $FoxP3^+$ cell infiltration has been reported to have no prognostic impact in patients with malignant melanoma or prostate cancer (325, 326). In SCLC, the local immune cell infiltrate and specifically the impact of infiltrating $FoxP3^+$ cells has not been extensively studied. In this chapter the presence of $FoxP3^+$ Treg cells within the primary

tumour microenvironment and the impact of Treg cell infiltration on patient survival are quantified for an SCLC cohort.

SCLC has a very poor prognosis despite treatment (10). Currently, only a small number of indicators, most of which are clinical markers (performance status, disease stage, age and sex) can be used to stratify patients with SCLC into prognostic groups (177). Early studies have demonstrated that some immune parameters may predict SCLC patient prognosis and disease stage. These include the degree of delayed hypersensitivity reaction and defects in peripheral blood lymphocyte numbers, proliferative response and IL-2 secretion (327-329). However, these studies have focused on the assessment of systemic immune responses that may not reflect the relevant changes in the local tumour environment. Immunohistochemical analyses of the local tumour inflammatory microenvironment in SCLC may reveal novel prognostic markers that should be simple and reproducible in a diagnostic laboratory. I undertook to profile immune cells within the tumour microenvironment in biopsies from a cohort of 64 SCLC patients.

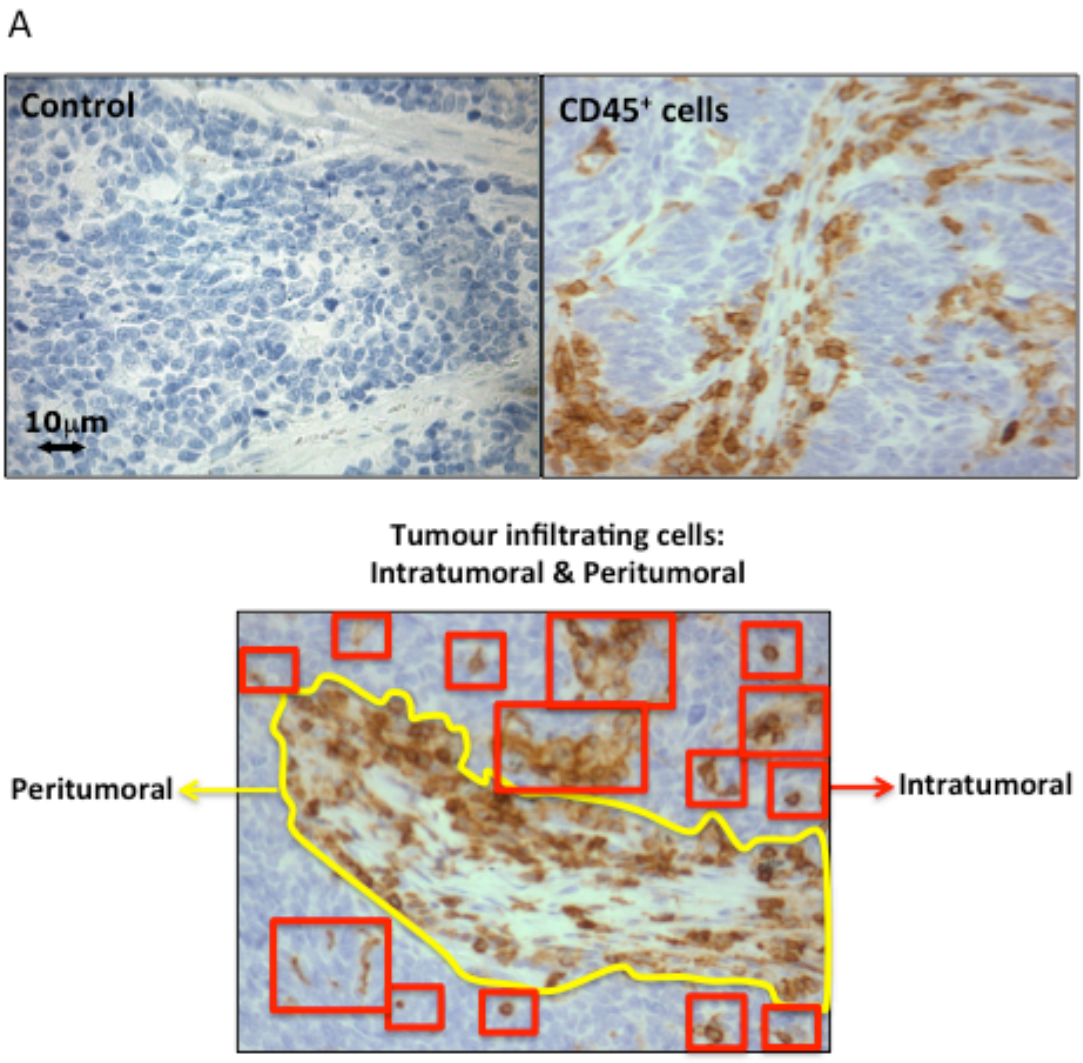
6.2 Results

6.2.1 SCLC tumour sections are infiltrated by various mononuclear immune cells.

In a pilot experiment to determine whether immune effector cells infiltrate SCLC and to identify the types, population and location of tumour-infiltrating immune cells, paraffin-embedded sections from 12 surgically resected SCLC tumours were immunohistochemically stained and viewed with light microscopy. Firstly, the leukocyte surface marker CD45 was used as a pan-inflammatory cell marker to stain mononuclear immune cells. CD45 did not stain SCLC tumour cells but $CD45^+$ cells were readily seen in tumour microenvironment in 12/12 patients (Fig. 6.1A). Tumour infiltrating $CD45^+$ cells were observed both within the tumour stroma (peritumoral) and in the tumour mass (intratumoral) (Fig. 6.1A).

To further characterize the cellular composition of $CD45^+$ infiltrates, CD3 (T cell marker), CD20 (B cell marker), CD14 (monocyte marker) and CD68 (macrophage marker) were used. $CD3^+$ cells were found in 12/12 SCLC tumour biopsy samples and were predominantly present in the tumour stroma, there were more $CD3^+$ cells within the stroma than the tumour mass (Fig. 6.1B). $CD4^+$ and $CD8^+$ T cells were identified in 12/12 SCLC sections. $CD4^+$ T cells showed a similar distribution to $CD3^+$ T cells in 12/12 patients. In contrast, 10/12 patients showed greater numbers of $CD8^+$ T cells infiltrating the tumour mass compared to peritumoral stroma (Fig. 6.1B). $CD20^+$ B cells were observed in low numbers compared to $CD3^+$ T cells, and were only present in the peritumoral stroma in 9/12 SCLC

tumour biopsies (Fig. 6.1B). 12/12 patients showed that $CD14^+$ monocytes and $CD68^+$ macrophages were both present within the tumour microenvironment. Fewer of these cells infiltrated the tumour mass compared to the peritumoral stroma (Fig. 6.1B).



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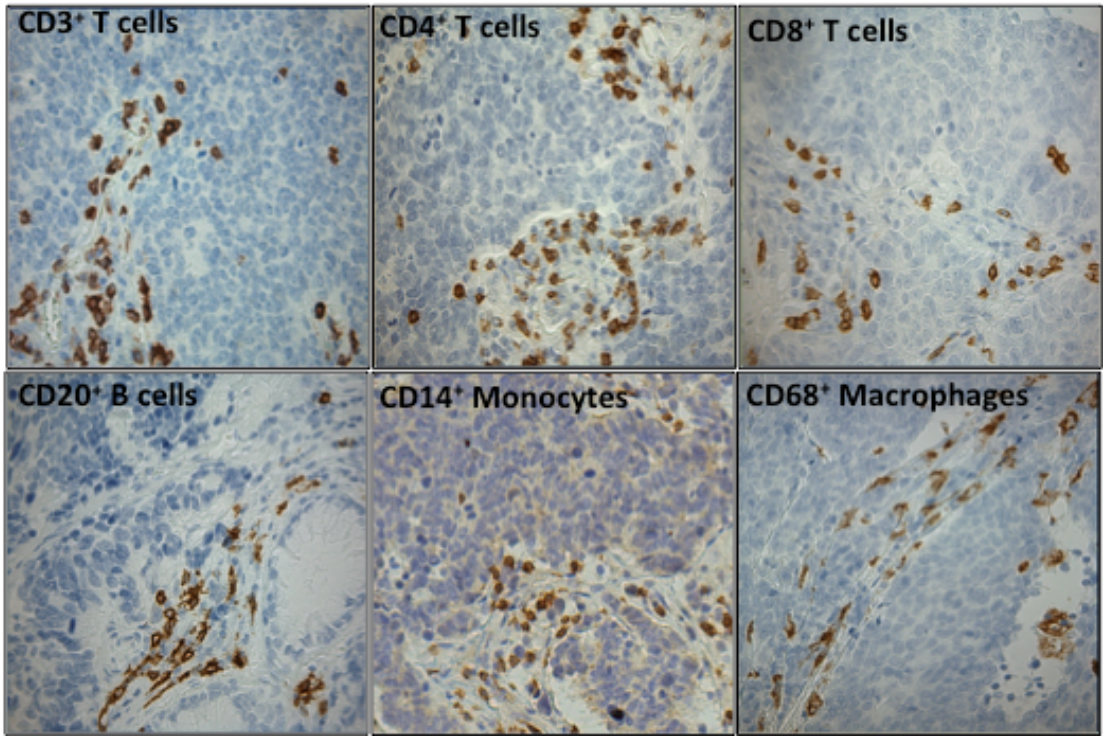

Figure 6.1: SCLC tumour sections are infiltrated by various mononuclear immune cells. Representative images of immunohistochemistry (IHC) showing (A) tumour infiltrating CD45 positive cells (intratumoral and peritumoral) and negative control. (B) CD3, CD4, CD8, CD20, CD14 and CD68 positive cells on formalin-fixed, paraffin-embedded tumour sections from SCLC patients.

6.2.2 SCLC tumour sections are infiltrated by FoxP3$^+$ Treg cells.

Tumour-infiltrating lymphocytes (TILs) include populations of both effector and Treg cells. A preponderance of Treg cells may lead to a failure of tumour immunosurveillance and contribute to the progression of cancer (330). FoxP3 staining was undertaken to determine the presence, population and distribution of Tregs in SCLC tumour microenvironment. CD3$^+$ T cells including CD3$^+$FoxP3$^+$

Treg cells and $CD3^+FoxP3^-$ effector cells were present in the tumour tissues, some $CD3^+$ T cells, but none of the tumour cells, were positive for nuclear FoxP3 (Fig. 6.2A). A number of $FoxP3^+$ cells were observed in the tumour stroma (peritumoral) but none in the tumour mass (intratumoral) (Fig. 6.2B). The data indicate that the presence of Treg cells in the tumour stroma may contribute immunosuppression and tumour progression.

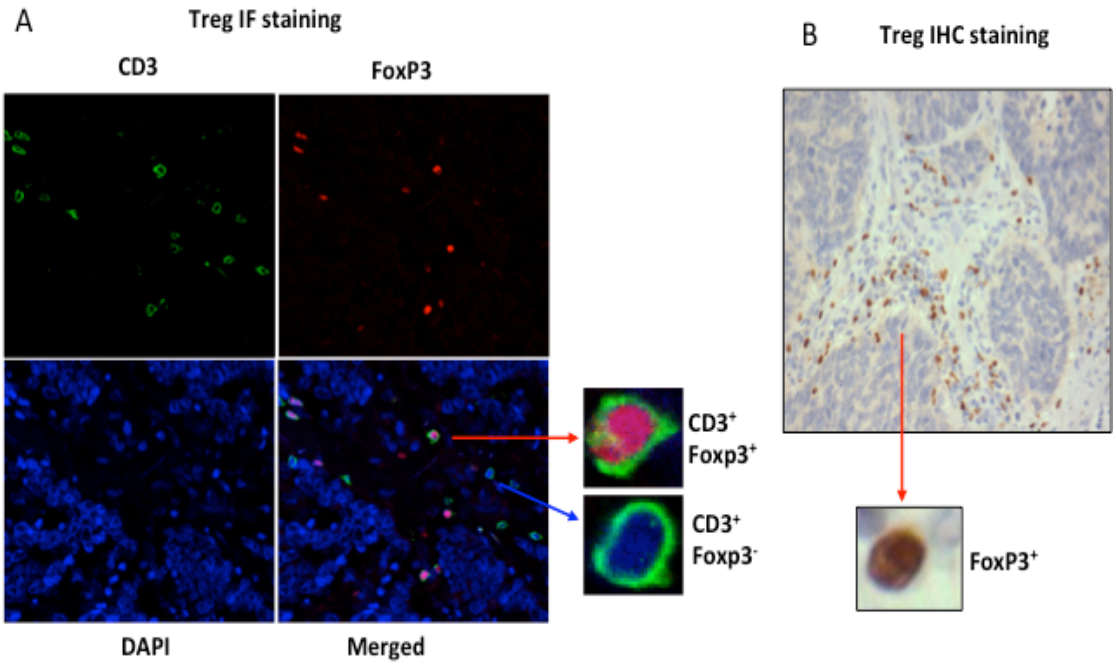

Figure 6.2 SCLC tumour sections are infiltrated by Treg cells**.** (A) Representative images of immunofluorescence (IF) staining showing SCLC sections simultaneously assessed for $CD3^+$ T cells, FoxP3 transcription factor, all nucleated cells (DAPI) and merged image. High power images of merged stains showing $CD3^+FoxP3^+$ and $CD3^+FoxP3^-$ cells isolated as indicated. (B) IHC showing $FoxP3^+$ cells on SCLC tumour sections.

6.2.3 FoxP3$^+$ cell infiltration does not predict patient survival in SCLC.

Considerable variability in the amount of FoxP3$^+$ cell infiltration among SCLC patients was observed, suggesting a biologically heterogeneous response (Fig. 6.3A). To determine whether FoxP3$^+$ cell infiltration may represent a prognostic marker of survival, IHC staining for FoxP3 on the tumour biopsies from 64 SCLC patients was performed. The details of study cases were collected and summarized (Table 6). The average number of FoxP3$^+$ cells per high-power field (hpf) was determined for each SCLC tumour sample. The positive cells were counted under X 400 magnification hpf/tumour sample, and the area of the field at this magnification was 0.18mm^2. 10 random high-power fields were counted per stain per biopsy and the score per field was averaged. Fields were not selected if they did not contain tumour cells. Analysis of interobserver variability showed no statistical differences between the mean counts achieved by the observers (Wei Wang and Prof T Sethi, King's College London, Dr W Wallace, University of Edinburgh).

The number of FoxP3$^+$ cells counted per field ranged from 1 to 40 with an average number of 8 per high-power field. Kaplan-Meier survival plots were devised by dividing the study group around the average value (8) (FoxP3 > 8, n=34; FoxP3 < 8, n=30). The patients with FoxP3$^+$ cell count > 8 did not show significant difference in survival compared with those with FoxP3$^+$ cell infiltration < 8 on tumour biopsies in SCLC (> 8 average of overall survival: 293.5 days; < 8 average overall survival: 230.5 days) (Fig. 6.3B). The data suggest that differences in

Foxp3^{+} Treg cell infiltration between patients may not represent a significant prognostic marker in SCLC.

Parameter		Study Cases
Age (year)		
	Median	66
	Range	38-80
Sex		
	Male	38
	Female	26
Stage		
	Limited	22
	Extensive	30
	Unknown	12
ECOG Permornace Status		
	0	7
	1	20
	2	13
	3	7
	4	4
	Unknown	13
Chemotherapy		
	Yes	41
	No	11
	Unknown	12
Radiotherapy		
	Yes	19
	No	33
	Unknown	12
Specimen type		
	Transthoracic biopsy	14
	Brochial biopsy	43
	Resection	4
	Mediastinal biopsy	3
Survival (day)		
	Median	232
	Range	4--3703

Table 6. Summary of details of SCLC study cases. Data are presented as number unless indicated otherwise. ECOG= Eastern Cooperative Oncology Group.

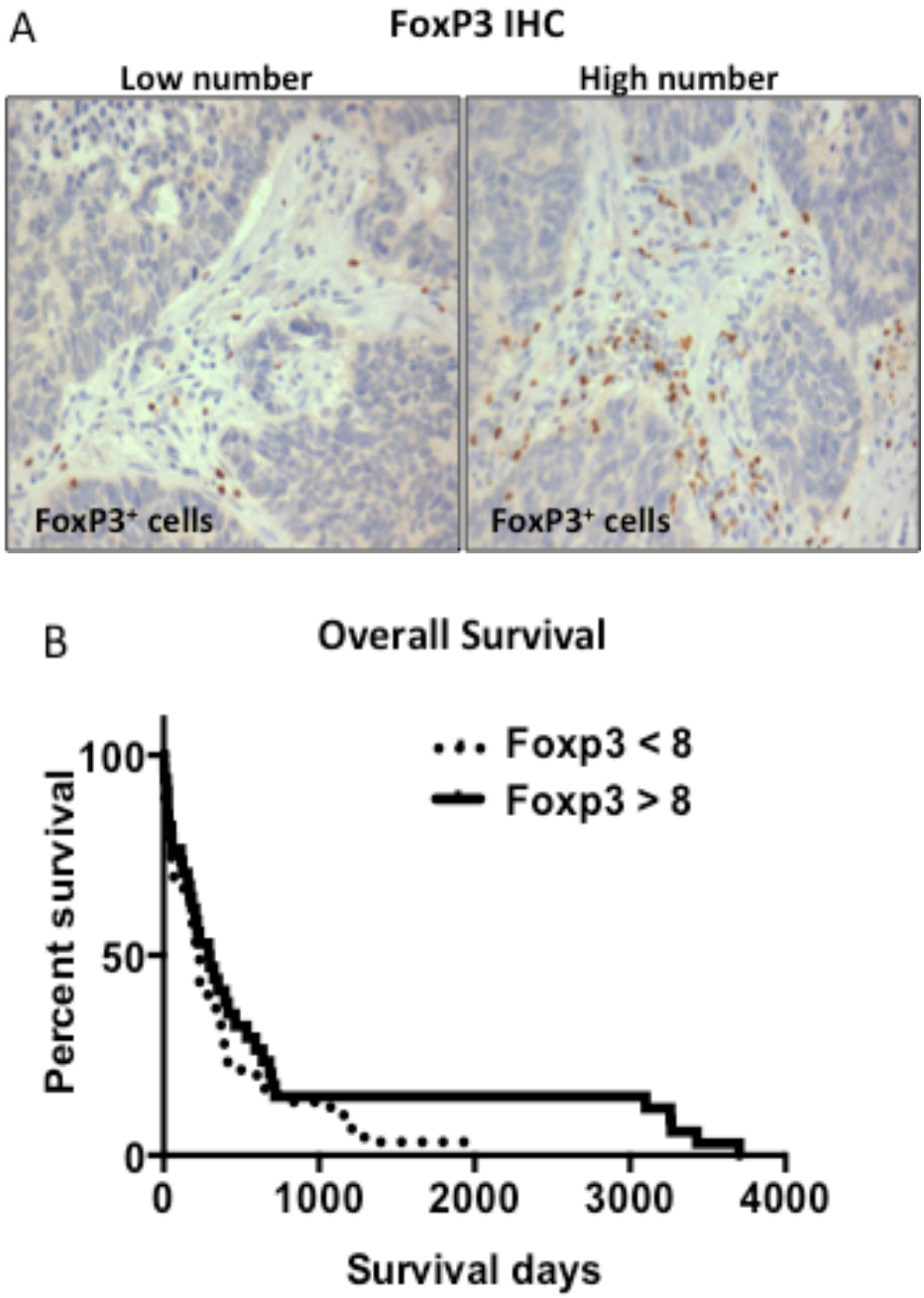

Figure 6.3: FoxP3$^+$ cell infiltration does not predict patient survival in SCLC. (A) Representative images of immunohistochemistry (IHC) staining showing low and high FoxP3$^+$ cell infiltration on tumour biopsies from SCLC patients. (B) Kaplan-Meier plots showing the survival of SCLC patients with FoxP3$^+$ cell count greater than or less than the average value of 8 cells per X400 magnification field.

6.2.4 $CD45^+$ cell infiltration is a predictor of survival in SCLC.

$CD45^+$ leukocytes were observed to be present in tumour site and the cell number varied between patients in SCLC (Fig. 6.4A). To determine whether $CD45^+$ cell infiltration represent a prognostic marker to predict survival in SCLC, IHC staining for CD45 on 64 SCLC tumour sections. Both intratumoral and peritumoral $CD45^+$ cells were counted as tumour infiltrates and CD45 positive cell counting per field per biopsy were performed as described above.

The number of $CD45^+$ cells counted per field ranged from 3 to 127 with an average value of 37.6 per high-power field. Univariate analysis of survival times was performed using Kaplan-Meier plots that were devised by dividing the study group around the average value (38) (CD45 > 38, n=30; CD45 < 38, n=34). The patients with $CD45^+$ cell number above the average survived significantly longer compared with those with $CD45^+$ cell infiltration less than 38 ($P < 0.0001$) (Fig. 6.4B). The average survival of patients in "high" CD45 count group was 534 days compared with the average survival time of 137.5 days in the group with fewer $CD45^+$ cells. Only 3% of the patients with a CD45 < 38 survived 1000 days compared with 27% of those with a CD45 > 38 ($P < 0.01$). No relationship between $CD45^+$ cell number and age ($P = 0.63$), sex ($P = 0.36$), stage ($P = 0.17$), or treatment by chemotherapy ($P = 0.12$) or radiotherapy ($P = 0.28$) was identified (χ^2 test). The data suggest that $CD45^+$ cell infiltration into tumour biopsy samples may be considered as a prognostic marker to predict survival in SCLC.

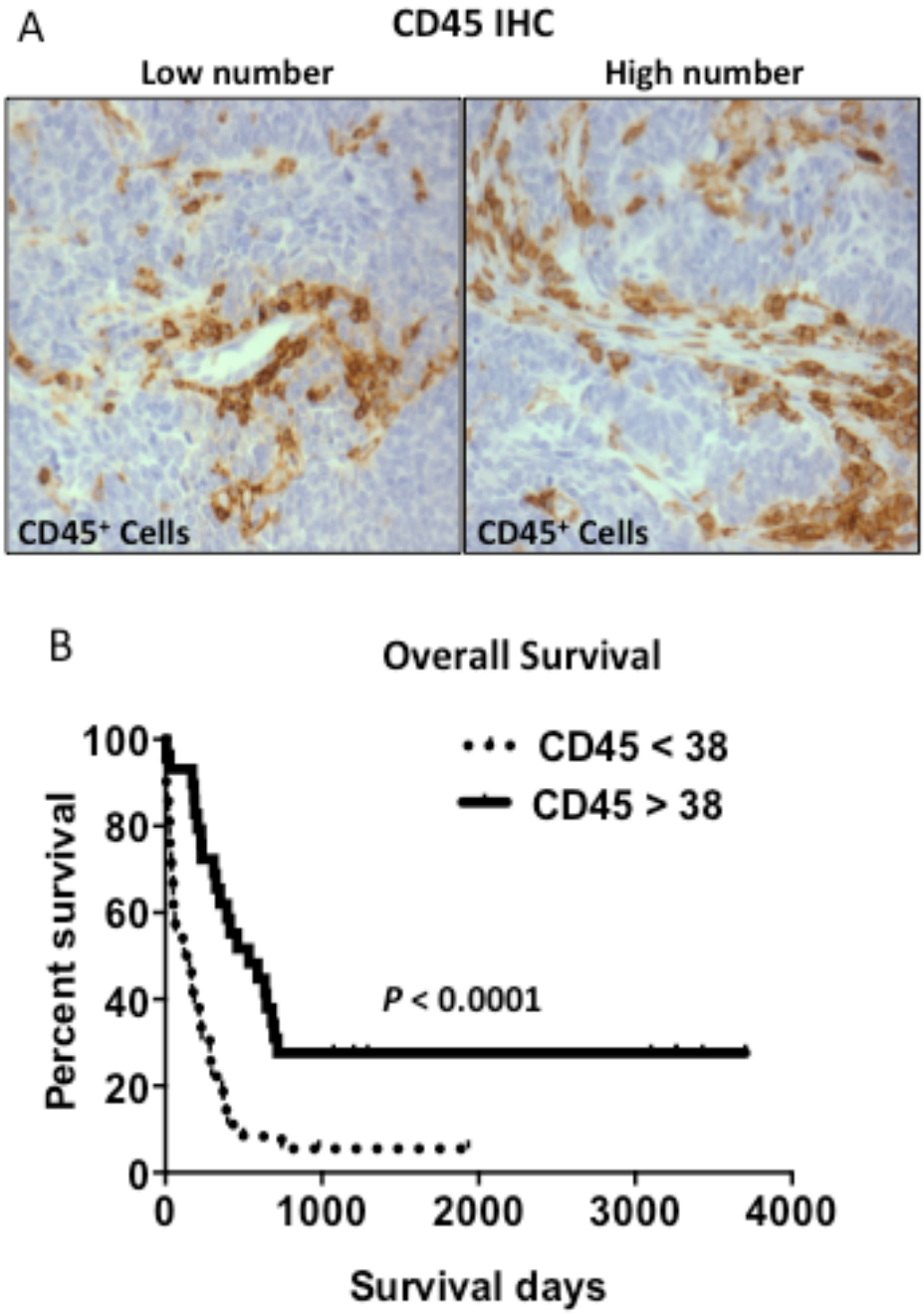

Figure 6.4: CD45$^+$ cell infiltration is a predictor of survival in SCLC. (A) Representative immunohistochemistry (IHC) showing low and high CD45$^+$ cell infiltration in tumour biopsies from SCLC patients. (B) Kaplan-Meier plots showing the survival of SCLC patients with CD45$^+$ cell count greater than or less than the median value of 38 cells per X400 magnification field.

6.2.5 The ratio of FoxP3$^+$/CD45$^+$ in SCLC tumour biopsy infiltrates negatively correlates with patient survival.

To determine the relationship between Treg cells in total leukocyte infiltrates of SCLC tumour biopsies and patient survival, the ratio of FoxP3$^+$: CD45$^+$ cells from the same biopsy was examined as a measure of proportion of Treg cells in the infiltrating leukocyte population. The ratios of FoxP3$^+$: CD45$^+$ cells from the 64 cases had a range from 3% to 80% with a median value of 28% per field per biopsy. The effect on patient survival time was determined using Kaplan-Meier analysis that was devised by dividing the study group around the median value (28%) (FoxP3/CD45 > 28%, n=31; FoxP3/CD45 < 28%, n=33). The patients with FoxP3$^+$: CD45$^+$ ratios above 28% had significantly worse overall survival compared with those with the ratios lower than 28% (P = 0.04) (Fig. 6.5). The average survival in “high” ratio group was 177.5 days compared with an average of 336.5 days in the group with the ratio less than 28%. The data indicate that the increased proportion of Treg cells in the leukocyte infiltrate within the tumour microenvironment is associated with poor survival in SCLC.

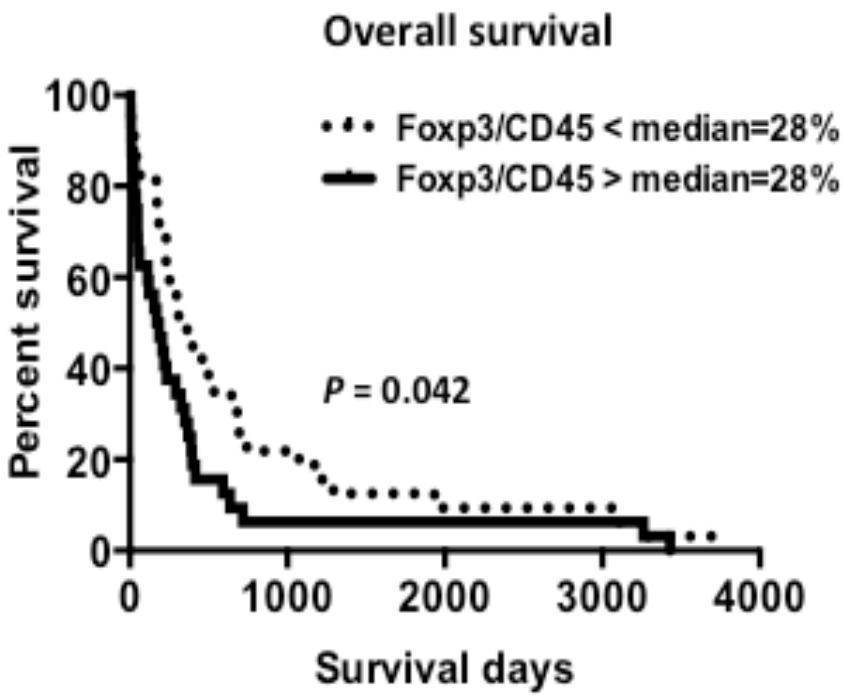

Figure 6.5: The ratio of FoxP3^{+}/CD45^{+} in SCLC tumour biopsy infiltrates negatively correlates with patient survival. Kaplan-Meier plots showing the survival of SCLC patients with the ratio of FoxP3^{+}/CD45^{+} on tumour biopsies greater than or less than the median value of 28% per X400 magnification field.

6.3 Discussion

The data in this chapter demonstrate that a number of immune cell types including $CD3^+FoxP3^+$ Tregs are present in the primary tumour microenvironment in SCLC. $FoxP3^+$ cell infiltration alone does not predict patient survival. However, patients with higher numbers of $CD45^+$ cells in tumour infiltrates have a highly significant survival advantage irrespective of disease stage, incidence of infection, sex, age and treatment. Therefore, $CD45^+$ cell infiltration in the tumour biopsies may represent an independent and significant prognostic marker to predict survival in SCLC. Furthermore, a relatively high proportion of $FoxP3^+$ Tregs in the total $CD45^+$ leukocyte infiltration negatively correlates with patient survival.

Previous studies in SCLC have demonstrated that high numbers of tumour infiltrating lymphocytes and macrophages are associated with small tumour sizes and improved patient survival (331). The same observations have been found in other cancers (313-317). These studies suggest the local immune cell infiltrate in tumour microenvironment may play a crucial role in control of cancer growth. The data presented here clearly demonstrate the presence of a $CD45^+$ mononuclear immune cell infiltrate in the human SCLC microenvironment. This leukocyte infiltrate consists of $CD3^+$ T cells ($CD4^+$ and $CD8^+$), $CD20^+$ B cells, $CD68^+$ macrophages and $CD14^+$ monocytes. The presence of these cells may represent a specialised and complex host immune response to the tumour cells in SCLC.

Histologic assessments of local immune responses to tumour as a prognostic marker have been proposed in many tumour types. However, there are no such

markers identified in SCLC. The data presented here indicate an early, steep fall in survival in the "low" $CD45^+$ cell number group. Patients with "high" numbers of $CD45^+$ infiltrate survive significantly longer, which may be associated with better host anti-tumour responses and thus may impair tumour growth and prolong survival. This is independent of other well-described prognostic markers including patient age, sex, stage or treatment. Therefore, the number of $CD45^+$ cells in the tumour microenvironment has a predictive effect on overall survival and can be regarded as an independent prognostic marker. Some previous studies in other tumour types have specifically identified infiltrating inflammatory cells as "intratumoral" or "peritumoral" in location (332, 333). In this study, $CD45^+$ cells in both locations are all considered as tumour infiltrating cells and could reasonably be regarded as "tumour associated". This is because the recruitment and retention of $CD45^+$ cells to the fragments of tissue at that site is likely related to the presence of the tumour. Importantly, the assessment of $CD45^+$ cell infiltrate as a prognostic marker by immunohistochemical stain in SCLC tumour biopsy samples can be routinely performed in a diagnostic laboratory. This method, being based on counting positive cells in high power field from fragments of tumour tissue, is relatively simple and reproducible, and may provide prognostic information without the requirement for additional complex investigations.

FoxP3 is considered as a marker of Treg cells (334). However, it has been shown that FoxP3 is expressed both at the transcript and protein level by tumour cells of various types (335). $FoxP3^+$ cells in SCLC tumour biopsies are confirmed as Treg cells by immunofluorescent double staining for CD3 and FoxP3, and SCLC tumour cells do not express FoxP3. $FoxP3^+$ cells are predominantly present in the tumour

stroma and very rarely infiltrate in tumour mass, similar to observations from other tumour types (325, 336). The amount of $FoxP3^+$ cells in tumour sections does not show association with any parameters of SCLC cases, and $FoxP3^+$ cell infiltration alone does not predict patient survival. Previous studies demonstrate contradictory results, showing that Treg cell infiltration in different cancers has been associated with all possible outcomes - poor survival (224, 319, 320), improved survival (321-324) and no correlation (325, 326). It may be that tumour types behave differently in this context (337), depending on other components of the complex anti-tumour immune responses and/or the interactions between tumour cells and Treg cells (224). These discordant observations suggest significant heterogeneity between different tumour types in terms of the impact of peritumoral Treg cells on patient outcome.

The distribution of Treg cells is different from $CD45^+$ immune cell infiltrates. Treg cells are restricted to the peritumoral stroma in SCLC while $CD45^+$ cells are in both peritumoral and intratumoral. These Tregs cells may reduce the recruitment of anti-tumour $CD45^+$ cells during tumour progression in SCLC. The suppressive effect on $CD45^+$ cell recruitment may be achieved by preventing immune effector cells from infiltrating to tumour sites and/or by suppressing proliferation of immune cells (211). Indeed, the patients with increased proportion of Treg cells in $CD45^+$ cell tumour infiltrates have a significantly worse survival rate. This may be, at least in part, because, the patients with higher $FoxP3^+$ ratios have a worse local anti-tumour immune response.

Chapter 7: General Discussion

Chapter 7 General Discussion

7.1 Conclusion

The general importance of immune surveillance in preventing cancer is indicated by the increased incidence of malignancy associated with immunosuppressive therapy (175, 176). Conversely, local and systemic immunosuppressive effects of tumour cells (106,107), including SCLC cells (160,161), mediate tumour tolerance. In SCLC such defects correlate with worse morbidity and mortality (160, 161). The work presented in this thesis support a range of immunomodulatory interactions between SCLC tumour cells and immune cells. Different SCLC tumour cell lines inhibit cell-mediated immune responses to differing extents through suppressing proliferation of a mixed leukocyte reaction and $CD4^+$ T cells (Chapter 3). Two important mechanisms by which SCLC tumour cells may suppress local and systemic immune responses have been identified. Firstly, SCLC cells can secrete IL-15 to induce $CD4^+$ T cells to adopt a functional Treg phenotype (Chapter 4). Secondly, SCLC cells can secrete BLPs to induce macrophage populations polarized towards an immunosuppressive, alternatively activated M2-like phenotype (Chapter 5). These novel findings may highlight potential new therapeutic targets to improve immune response and patient survival in SCLC.

SCLC has very poor prognosis and only a small number of indicators can be used to stratify SCLC patients into prognostic groups (177). These include clinical markers such as performance status (PS), disease stage (LD or ED), age, and sex (177). Histologic assessments of local immune responses to tumour as a marker of prognosis have been studied in this work. $CD45^+$

immune cells and FoxP3$^+$ Treg cells are found in tumour biopsies in SCLC patients (Chapter 6). CD45$^+$ cell tumour infiltrates have a prognostic effect on patient survival that is independent of age, sex, stage, or treatment (Chapter 6). Therefore, it may represent an independent and significant prognostic marker in SCLC. The proportion of FoxP3$^+$ Treg cell: total CD45$^+$ leukocyte within tumour microenvironment (but not absolute FoxP3$^+$ cell count alone) negatively correlates with patient survival in SCLC. These novel findings have defined simple and reproducible prognostic markers that may assist medical decision making and improve future research design.

7.2 Identification of factor(s) that can induce the Treg population to suppress CD4$^+$ T cell proliferation.

The data presented in Chapters 3 and 4 demonstrate that three different SCLC cell lines have differential immunosuppressive effects on MLR and CD4$^+$ T cell proliferation and induction of functional FoxP3$^+$ Treg cells. These effects are in a consistent order H510<H345<H69 and may be associated with the levels of soluble factors that are constitutively produced by SCLC cells. These molecules are not IL-10 or TGF-β as neutralization of both fail to reverse the suppressive effect on T cell proliferation. BLPs produced by H69 cells are ruled out in Chapter 7, showing that blocking BLP signalling has no effect on suppression. IL-15 has been identified as one, but not the only, soluble factor produced by H69 cells that can mediate immune suppression. This is because blocking of IL-15 activity only partially reverses the suppression of CD4$^+$ T cell proliferation

and induction of Treg cells. Furthermore, the combination of IL-15 and H510CM induces similar Treg cell population to IL-15 alone and less than H69CM. These data indicate that H69 cells secrete other soluble factors that are involved in Treg cell induction and that are not secreted by H510 cells. In addition to IL-15, a number of cytokine genes (IL-1α, IL-11, IL-16, BMP-7 and CSF-2) are up-regulated in H69 cells relative to H510 cells. The effects of these cytokines on Treg cell induction should be the focus of future experiments.

In addition to inhibitory cytokines (IL-10 and TGF-β), other immunosuppressive factors derived from tumour cells such as indoleamine 2,3-dioxygenase (IDO), vascular endothelial growth factor (VEGF), adenosine and galectins, have been described in other cancers (332). High levels of IDO1 produced by tumour cells can induce FoxP3^{+} Treg cell population and directly suppress T cell activity (339). The presence of VEGF may induce immunosuppression by affecting the maturation of DCs and macrophages (340). Adenosine is secreted by tumour cells and immune cells during tumor pathogenesis. It can exert immunomodulatory effects via its different receptors expressed on various immune cells including Treg cells (341). Moreover, galectins produced by tumour cells can suppress anti-tumour responses by affecting phenotype and function of various immune cells (342). Assessments of the presence and effects of these factors may be therefore usefully further investigated in SCLC.

7.3 The effects of IL-15 in anti-cancer immune responses.

The role of IL-15 in human cancers is complex. IL-15 can either function as an immune-stimulatory cytokine to boost host immune responses or function as a growth factor to promote tumour cell proliferation and migration (343-345). IL-15 is produced by a variety of cells and has various functions in regulation of immune responses. These functions include stimulating the proliferation, differentiation and activation of NK cells, NK-T cells, $CD4^+$ and $CD8^+$ T cytotoxic cells, B cells, monocytes and APCs (254, 346). Therefore, IL-15 has been promoted as a potential anti-tumour therapy to boost immune responses against cancers, and some clinical trials using IL-15 are being performed (343-345). In fact, IL-15 used alone, or in combination with other treatment modalities, has been shown to improve immune responses in solid tumours. However, IL-15 has been found to be associated with inflammation that may be linked to cancer pathogenesis (346). Furthermore, IL-15 can also act as a growth factor to inhibit apoptosis of tumour cells and promote their proliferation and migration, thereby leading to tumour progression and metastasis (343). Therefore, the use of IL-15 in cancer immunotherapy requires some caution, at least in principle, and its effects upon the specifically targeted tumour cells should be examined carefully in different cancers.

Despite a growing interest in the use of IL-15 as an immunotherapeutic agent, the broad spectrum of immunomodulatory functions exerted by IL-15 has not been fully elucidated. IL-15 and IL-2 have a number of similar functions to potentiate the immune system (344). However, IL-15 has pleiotropic effects on

Treg cells in contrast to IL-2, which plays a critical role in the differentiation and maintenance of FoxP3$^+$ Treg cells (338). The data presented in Chapter 4 demonstrate that SCLC cell derived-IL-15 may play a role in SCLC pathogenesis to induce Treg cells that suppress CD4$^+$ T cell proliferation. This is consistent with other studies showing that IL-15 can induce a population of FoxP3$^+$ Treg cells (235, 257-260). On the other hand, IL-15 can render effector T cells resistant to suppressive effects of Treg cells and reverse the tolerance of effector T cells to tumour-associated antigens (TAAs) (343). Induction of Treg cells that induce tolerance to TAAs and suppress the anti-tumour immune responses represents a major obstacle to the development of effective tumour immunotherapies (176). Therefore, reduction of Treg cell population in tumour sites may improve cancer immunotherapy. In SCLC, IL-15 may represent a potential new therapeutic target to improve immune response and patient survival.

7.4 The mechanisms of SCLC cell-induced Treg cell suppression.

It is well established that iTregs can exert their suppressive activity on effector T cells by secreting large amounts of the potent immunosuppressive cytokines IL-10 and TGF-β (214). The data in Chapters 3 and 4 demonstrate that SCLC cells induce an increased population of Treg cells by adaptive induction from naïve CD4$^+$ T cells. These iTreg cells can suppress the proliferation of effector CD4$^+$ T cells (Chapter 4). However, the suppressive effect of SCLC cell-induced Treg cells is not mediated through the production of immunosuppressive cytokines IL-10 or TGF-β (Chapters 3 and 4). This suggests that SCLC cells may induce a

distinct subtype of iTregs relative to those have previously been described. Future experiments may need to investigate the mechanisms of SCLC cell-induced Treg cell suppression and the phenotype of the resultant iTregs.

A number of possible alternative mechanisms may underlie the immunomodulatory effects of such iTreg cells (239). These include pathways mediated by IL-35 and granzymes. IL-35, a new member of IL-12 cytokine family, has been discovered as a new inhibitory cytokine that can that can directly inhibit effector T cell proliferation in mouse (347). Human Treg cells do not constitutively express IL-35 (348). Nevertheless treatment of naïve human T cells with IL-35 induces Treg cells with suppressive effects by stimulating IL-35 production and this process does not require IL-10, TGF-β or FoxP3 (349). *In vitro* studies indicate these Treg cells need to be activated for a prolonged period to exert suppressive effects, and that these mechanisms are independent of direct cell contact (350). Future experiments can be performed to determine whether blocking of IL-35 has effects on Treg cell induction and the suppression of $CD4^+$ T cell proliferation.

Granzymes are serine proteases, secreted mainly by NK cells and CTLs. They are largely responsible for the induction of apoptosis in target cells (351). The perforin/granzyme pathway is one of the key mechanisms by which Treg cells mediate immune suppression (239). Activated Treg cells are capable of killing autologous target cells in a cell contact-mediated and perforin-dependent manner. Specific granzymes are utilized by nTregs (granzyme A) and iTregs (granzyme B) (342, 353). Similarly, in mice, Treg cells derived from the tumour

environment highly express granzyme B, and mediate immune suppression in a granzyme B and perforin-dependent fashion *in vivo* (354). Interestingly, in mice, it has also been shown that contact-mediated suppression by Treg cells involves a granzyme B-dependent, perforin-independent mechanism (355). Future experiments can be performed to determine the expression of granzyme A/B in SCLC cell-induced Tregs, whether these cells are cytotoxic to autologous effector $CD4^+$ T cells and the extent to which such effects are perforin-dependent.

7.5 The phenotype and function of tumour-associated macrophages (TAMs) in SCLC.

Inflammatory cells and mediators are important constituents of the local tumour microenvironment (356). Tumour-associated macrophages (TAMs) are a major component of the host immune cell infiltrate of tumours, and the recruitment of circulating monocytes is essential for TAM infiltration (144). Tumoricidal M1-like and pro-tumoral M2-like macrophages are the main two types of TAMs with diverse phenotypes and functions, they both significantly correlate with tumour initiation and progression (143, 144). Previous studies have suggested that TAMs could be either M1- or M2-like macrophages (357). *In vitro* studies in this thesis demonstrate that upon activation, monocyte-derived macrophages (MDM) have a mixed phenotype expressing both M1 and M2 markers (Chapter 5). However, they are polarized to an M2-like phenotype by SCLC cell-derived BLPs. Specifically, the polarization is associated with reduced expression of M1

markers (CD16/CD86) and increased expression of M2 markers (CD163/CD206). In addition, the polarization is accompanied by a reduction in pro-inflammatory cytokine secretion (TNF-α/IL-6) and an increase in immunosuppressive cytokine production (IL-10) (Fig. 7). Therefore, SCLC cells have the potential to induce immunosuppressive populations of TAMs within the tumour microenvironment.

However, the phenotype and function of TAMs *in vitro* may not directly reflect their plasticity *in vivo* within the complex tumour microenvironment. Accurate identification and characterization of these various populations of TAMs within the tumour microenvironment may highlight therapeutic targets and predict prognosis. It has become clear that TAMs are composed of multiple distinct populations with overlapping features. These depend on a variety of external factors including characters of activation in the complex tumour microenvironment, stage and type of cancers (143, 358). Studies of NSCLC tumour biopsies have revealed that the TAMs are composed of both M1 and M2 macrophages (359, 360). The presence of the M1 form in TAMs is positively associated with patient survival, whilst M2 macrophages with immunosuppressive function are predominant population of TAMs and the M2 density also correlate patient survival (359, 360).

Previous studies have tried to relate TAM infiltration to prognosis in human lung cancer, with various results including positive, negative and no correlation (361). In SCLC, these have not been extensively studies. The data presented here demonstrate the presence of $CD68^+$ macrophages in tumour biopsy samples

from SCLC patients. Future experiments are required to investigate the phenotype of TAMs in SCLC tumours *in vivo* using multiple phenotypic markers, and to determine their effects on patient survival in SCLC. Future studies may then usefully investigate their effects on proliferation of $CD4^+$ T cells and induction of Treg cells.

7.6 Causes and implications of differential immune cell infiltration in SCLC *in vivo.*

7.6.1 Differential immunosuppression by SCLC in different patients may account for $CD45^+$ cell infiltrate variability.

The data presented in chapters 4 and 5 demonstrate that IL-15 and BLPs produced by SCLC cells *in vitro* can induce functional Treg cells and immunosuppressive M2 macrophages respectively. Other factors produced by SCLC cells may also contribute to the suppressive effects on anti-tumour immune responses. *In vivo* production of these factors may suppress the local host immune responses by acting upon different types of $CD45^+$ cells. Differences in the production of these factors within the tumour microenvironment would thereby affect the recruitment and retention of immune cells. This may explain the observed differences in $CD45^+$ leukocyte infiltrates and patient survival. Indeed, the data in chapter 5 demonstrate that the proportion of $Foxp3^+$ Treg cells to $CD45^+$ leukocytes in tumour infiltrates significantly correlates with patient survival. Future experiments usefully

investigate IL-15 and BLP expression in SCLC tumour samples and correlate the expression with immune cell infiltrates and patient survival.

7.6.2 $CD45^+$ cell number within tumour microenvironment is considered as an independent prognostic marker.

Histologic assessments of SCLC tumour biopsies indicate that the presence of $CD45^+$ cells within the tumour microenvironment may represent a specific anti-tumour immune response (Chapter 6). $CD45^+$ cell infiltration within the tumour microenvironment may eradicate malignant cells and control tumour growth, and the patients should benefit in survival. If so, then patient survival may correlate with $CD45^+$ cell infiltration into tumour tissues. I have studied this in a cohort of 64 typical SCLC patients for whom clinical details are available. The *ex vivo* data support this hypothesis. Patients with higher numbers of $CD45^+$ cells within the tumour microenvironment have a highly significant survival advantage irrespective of disease stage, incidence of infection, sex, age and treatment. The study is relatively small, and retrospective. It may be built upon by future studies by assessing more SCLC tumour samples prospectively by immunohistochemical methods to assess $CD45^+$ cell numbers. In addition, the biopsies should be stained with Ki67 (proliferation marker), CD34 (angiogenesis marker) and TUNEL (apoptosis marker), to determine the effects of $CD45^+$ cell infiltration on tumour cell growth.

7.6.3 Identification of other immune cell infiltrates as prognostic markers.

Different types of immune cells are recruited to the tumour sites, their distribution, tissue localization and function are significantly associated with tumour progression and patient survival (362). Various mononuclear immune cells have been found in SCLC tumour biopsy samples (Chapter 6). Assessment of individual immune cell types within tumour microenvironments may be more accurate than assessment of total $CD45^+$ inflammatory cells in prediction of patient prognosis. $CD4^+$ T cells play a critical role in co-ordinating tumour rejection (174). The data presented in chapters 3 and 4 demonstrate that SCLC cells can induce expansion of Treg cells to suppress $CD4^+$ T cell proliferation *in vitro*. Therefore, future studies should assess the presence of $CD4^+$ T cells within the tumour microenvironment that may correlate accurately with improved patient survival. The data in chapter 6 indicate that $FoxP3^+$ cell number has no prognostic effect to predict patient survival, except when considered as a proportion of $CD45^+$ cell numbers. In comparison, studies in other cancers demonstrate that the effect of $FoxP3^+$ cells on patient survival is controversial. I believe that $FoxP3^+$ cell infiltration as a prognostic marker may be tumour-dependent and it may not be accurately used in SCLC. TILs including $CD8^+$ T cytotoxic cells, and TAMs including M1 and M2 macrophages, have been reported to predict prognosis in other cancers with various results. However, the relationship between these immune cell infiltrates and prognosis has not been examined extensively in SCLC. Future experiments may aim to collect more SCLC biopsies to identify the number and distribution of these cells within the tumour microenvironment, and correlate with patient survival and

tumour stage. These investigations would provide *in vivo* data to correlate with the observations from *in vitro* studies, and validate further study of potential targets for anti-cancer therapy.

7.7 Overall model arising from this work and implications for future studies.

In this thesis two cell-mediated mechanisms by which SCLC cells may suppress immune responses have been identified. Firstly, SCLC cells secrete IL-15 to induce $CD4^+$ T cells to adopt a functional Treg phenotype, and patient survival correlates negatively with proportion of these cells ($CD3^+FoxP3^+$) in tumour infiltrate (Fig. 7). However, IL-15 may be an important factor secreted by SCLC cells to mediate immunosuppression though not the only such factor. Future studies should identify the other immunosuppressive factors. In addition, the suppressive mechanism by which these iTreg cells function is independent of IL-10 and TGF-β. Future experiments may therefore usefully investigate the mechanisms by which SCLC cell-induced Treg cells suppress $CD4^+$ T cell proliferation. The second mechanism is that SCLC cells can secrete BLPs to induce macrophage populations polarized to an alternatively activated M2-like phenotype (Fig. 7). Future experiments may usefully investigate their effects on proliferation of $CD4^+$ T cells and induction of Treg cells. Moreover, the populations of M1- and M2-like macrophages in SCLC tumour infiltrates and their effects on patient survival may be determined in future studies.

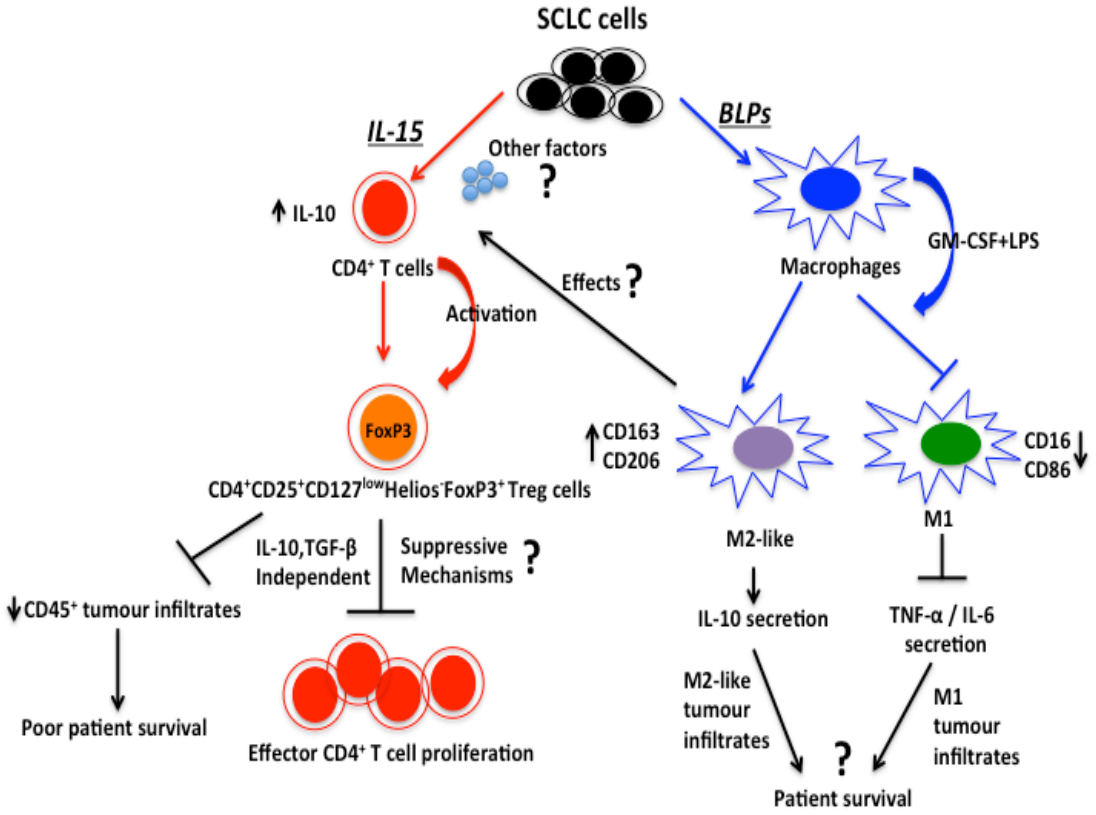

Figure 7: Model scheme of immune suppressive effects of SCLC cells. The immune suppressive effects of SCLC cells on $CD4^+$ T cells and macrophages. 1) SCLC cells produce IL-15 to induce $CD4^+$ T cells to adopt $CD4^+CD25^+CD127^{low}Helios^-FoxP3^+$ Treg cells upon activation. The induction of Tregs is associated with increased IL-10 release. These iTreg cells can suppress effector CD4+ T cell proliferation independent of IL-10 or TGF-β. Analysis of tumour biopsy samples demonstrates that the proportion of these $FoxP3^+$ cells in tumour infiltrate negatively correlates with patient survival in SCLC. Future studies should identify the other immunosuppressive factors and suppressive mechanism of iTreg cells. 2) SCLC cells produce BLPs to induce macrophage populations polarized to an alternatively activated M2-like phenotype. The polarization is associated with increased expression of CD163 and CD206, and decreased expression of CD16 and CD86. IL-10 production is increased and TNF-α/IL-6 production is reduced. Future experiments may investigate their effects on proliferation of $CD4^+$ T cells and induction of Treg cells. The populations of M1- and M2-like macrophages in SCLC tumour infiltrates and their effects on patient survival may be determined in future studies.

Chapter 8: References

1. Lemjabbar-Alaoui H, Hassan OU, Yang YW, Buchanan P. Lung cancer: Biology and treatment options. Biochim Biophys Acta. 2015; 1856(2):189-210.
2. Feraly J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015; 136(5):E359-86.
3. Herbst RS, Heymach JV, Lippman SM. Lung Cancer. N Engl J Med 2008; 359:1367-80.
4. Chen Z, Fillmore CM, Hammerman PS, Kim CF, Wong KK. Non-small-cell lung cancers: a heterogeneous set of diseases. Nat Rev Cancer. 2014; 14(8):535-46.
5. Langer CJ, Besse B, Gualberto A, Brambilla E, Soria, JC. The evolving role of histology in the management of advanced non–small-cell lung cancer. J. Clin. Oncol. 2010; 28:5311–5320.
6. Davidson MR, Gazdar AF, Clarke BE. The pivotal role of pathology in the management of lung cancer. J. Thorac. Dis. 2013; 5:S463–S478.
7. Ettinger DS, Akerley W, Borghaei H, Chang AC, Cheney RT, Chirleac LR et al. Non-Small Cell Lung Cancer, Version 2.2013. J. Natl Compr. Canc. Netw. 2013; 11:645–653.
8. Ohe Y. Chemoradiotherapy for lung cancer: current status and perspectives. Int J Clin Oncol 2004; 9:435-443.
9. Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC, editors. Pathology and genetics of tumours of the lung, pleura, thymus and heart. Lyon: IARC Press; 2004. World Health Organization *Classification of tumours;* pp. 31–4.

10. El Maalouf G, Rodier JM, Faivre S, Raymond E. Could we expect to improve survival in small cell lung cancer? Lung Cancer. 2007; 57 Suppl 2:S30-4.
11. Rintoul RC, Sethi T. The role of extracellular matrix in small-cell lung cancer. Lancet Oncol 2001; 2:437–42.
12. Oze I, Hotta K, Klura K, Ochi N, Takigawa N, Fujiwara Y et al. Twenty-seven years of phase III trials for patients with extensive disease small-cell lung cancer: disappointing results. PLoS One 2009; 4:e7835.
13. de Groot P, Munden RF. Lung cancer epidemiology, risk factors, and prevention. Radiol Clin North Am 2012; 50:863-876.
14. Alberg AJ, Samet JM. Epidemiology of lung cancer. Chest. 2003; 123(1 Suppl):21S-49S.
15. Toh CK, Gao F, Lim WT, Leong SS, Fong KW, Yap SP et al. Differences between small-cell lung cancer and non-small-cell lung cancer among tobacco smokers. Lung Cancer. 2007; 56(2):161-6.
16. De Stefani E, Boffetta P, Ronco AL, Brennan P, Correa P, Deneo-Pellegrini H et al. Squamous and small cell carcinomas of the lung: similarities and differences concerning the role of tobacco smoking. Lung Cancer. 2005; 47(1):1-8.
17. Gustafsson BI, Kidd M, Chan A, Malfertheiner MV, Modlin IM. Bronchopulmonary neuroendocrine tumors. Cancer. 2008; 113(1):5-21.
18. Dearing MP, Steinberg SM, Phelps R, Anderson MJ, Muishine JL, Ihde DC et al. Outcome of patients with small-cell lung cancer: effect of changes in staging procedures and imaging technology on prognostic factors over 14 years. J Clin Oncol. 1990; 8(6):1042-9.

19. Marchioli CC, Graziano SL. Paraneoplastic syndromes associated with small cell lung cancer. Chest Surg Clin N Am. 1997; 7(1):65-80.
20. Rivera MP, Detterbeck F, Mehta AC. Diagnosis of lung cancer: the guidelines. Chest. 2003; 123(1 suppl):129S–136S.
21. Travis W, Brambilla E, Nicholson AG, Yatabe Y, Austin JHM, Beasley MB et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances Since the 2004 Classification. Journal of Thoracic Oncology. 2015; Volume 10 - Issue 9 - p 1243–1260.
22. Mountain CF. Revisions in the International System for Staging Lung Cancer. Chest. 1997; 111(6):1710-7.
23. Argiris A, Murren JR. Staging and clinical prognostic factors for small-cell lung cancer. Cancer J. 2001; 7(5):437-47.
24. Ganti AK, West WW, Zhen W. Current concepts in the management of small cell lung cancer. Indian J Med Res. 2013; 137(6):1043-1051.
25. Anraku M, Waddell TK. Surgery for small-cell lung cancer. Semin Thorac Cardiovasc Surg. 2006; 18:211–216.
26. Carmichael J, Mitchell JB, DeGraff WG, Gamson J, Gazdar AF, Johnson BE et al. Chemosensitivity testing of human lung cancer cell lines using the MTT assay. Br J Cancer. 1988;57(6):540-7.
27. Raez L, Samuels M, Lilenbaum R. Combined modality therapy for limited disease small cell lung cancer. Curr. Treat. Options Oncol. 2005; 6: 69–74.
28. Kelly K. New Chemotherapy Agents for Small Cell Lung Cancer. Chest. 2000; 117 (4 Suppl 1):156S-162S.

29. Kurup A, Hanna NH. Treatment of small cell lung cancer. Crit. Rev. Oncol. Haematol. 2004; 52: 117–26.

30. Mascaux C, Paesmans M, Berghmans T, Branie F, Lafitte JJ, Lemaitre F et al. A systematic review of the role of etoposide and cisplatin in the chemotherapy of small cell lung cancer with methodology assessment and meta-analysis. Lung Cancer 2000; 30: 23–36.

31. Kosmidis PA, Samantas E, Fountzilas G, Pavlidis N, Apostolopoulou F, Skarlos D. Cisplatin/etoposide versus carboplatin/etoposide chemotherapy and irradiation in small cell lung cancer: a randomized Phase III study. Hellenic Coorporation Oncology Group for Lung Cancer Trials. Semin. Oncol. 1994; 21: 23–30.

32. Noda K, Nishiwaki Y, Kawahara M, Negoro S, Sugiura T, Yokoyama Akira et al. for the Japan Clinical Oncology Group. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. N. Engl. J. Med. 2002; 346: 85–91.

33. Horiike A, Saijo N. Small cell lung cancer: current therapy and novel agents. Oncology (Williston Park) 2005;19: 47–52.

34. Han JY, Cho KH, Lee DH, Kim HY, Kim EA Lee SY et al. Phase II study of irinotecan plus cisplatin induction follwed by concurrent twice-daily thoracic irradiation with etoposide plus cisplatin chemotherapy for limited disease small cell lung cancer. J. Clin. Oncol. 2005; 23: 3488–94.

35. Saijo N, Horiike A. Topoisomerase I inhibitors in small cell lung cancer. The Japanese experience. Oncology (Williston Park) 2004; 8: 11–16.

36. Hanna N, Bunn PA Jr, Langer C, Einhorn L, Guthrie T Jr, Beck T et al. Randomized phase III trial comparing irinotecan/cisplatin with

etoposide/cisplatin in patients with previously untreated extensive-stage disease small-cell lung cancer. J Clin Oncol. 2006; 24(13):2038-43.

37. Mavroudis D, Papadakis E, Vesiemes M, Tsiafaki X, Stavrakakis J, Kouroussis C et al. A multicenter randomized clinical trial comparing paclitaxel-cisplatin-etoposide versus cisplatin-etoposide as first-line treatment in patients with small-cell lung cancer. Ann Oncol. 2001; 12(4):463-70.
38. Cooper S, Spiro SG. Small cell lung cancer: Treatment review. Respirology. 2006; 11, 241–248.
39. Carmichael J, Degraff WG, Gamson J, Russo D, Gazdar AF, Levitt ML et al. Radiation sensitivity of human lung cancer cell lines. Eur J Cancer Clin Oncol. 1989; 25(3):527-34.
40. Warde P, Payne D. Does thoracic irradiation improve survival and local control in limited-stage small-cell carcinoma of the lung? A meta-analysis. J Clin Oncol. 1992; 6:890-5
41. Pignon JP, Arriagada R, Ihde DC. A meta-analysis of thoracic radiotherapy for small-cell lung cancer. N Engl J Med. 1992; 23:1618-24.
42. Pijls-Johannesma M, De Ruysscher D, Vansteenkiste J, Kester A, Rutten I, Lambin P. Timing of chest radiotherapy in patients with limited stage small cell lung cancer: a systematic review and meta-analysis of randomised controlled trials. Cancer Treat Rev. 2007; 33(5):461-73.
43. Turrisi AT III, Kim K, Blum R. Twice-daily compared with once-daily thoracic radiotherapy in limited small-cell lung cancer treated concurrently with cisplatin and etoposide. N Engl J Med 1999; 340:265-71.

44. Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer 2003;3:733–744.

45. Gazdar AF, Minna JD. Cigarettes, sex, and lung adenocarcinoma. J. Natl. Cancer Inst. 1997; 89:1563–65

46. Li D, Firozi PF, Wang LE, Bosken CH, Spitz MR, Hong WK et al. Sensitivity to DNA damage induced by benzo(a)pyrene diol epoxide and risk of lung cancer: a case-control analysis. Cancer Res. 2001; 61:1445–50.

47. Tischfleld JA. Loss of heterozygosity or: how I learned to stop worrying and love mitotic recombination. Am J Hum Genet. 1997; 61(5): 995-9.

48. Wistuba II, Behrens C, Virmani AK. High resolution chromosome 3p allelotyping of human lung cancer and preneoplastic/preinvasive bronchial epithelium reveals multiple, discontinuous sites of 3p allele loss and three regions of frequent breakpoints. Cancer Res. 2000; 60:1949-1960.

49. Kok K, Naylor SL, Buys CH. Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. Adv Cancer Res. 1997; 71:27-92.

50. Sozzi G, Veronese ML, Negrini M, Baffa R, Cottichlli G, Inoue H et al. The FHIT gene 3p14.2 is abnormal in lung cancer. Cell. 1996; 85(1):17-26.

51. Miura I, Graziano SL, Cheng JQ, Doyle LA, Testa JR. Chromosome alterations in human small cell lung cancer: frequent involvement of 5q. Cancer Res. 1992; 52(5):1322-8.

52. D'Angelo SP, Pietanza MC. The molecular pathogenesis of small cell lung cancer. Cancer Biol Ther. 2010; 10(1): 1-10.

53. Shen C, Wang X, Tian L, Che G. Microsatellite alteration in multiple primary lung cancer. J Thorac Dis. 2014; 6(10): 1499-1505.
54. Sekido Y, Fong KM, Minna JD. Progress in understanding the molecular pathogenesis of human lung cancer. Biochem Biophys Acta, 1998; 1378, pp. F21–F59.
55. Hodkinson PS, Mackinnon A, Sethi T. Targeting growth factors in lung cancer. Chest. 2008; 133(5):1209-16.
56. Zhou J, Chen J, Mokotoff M, Ball ED. Targeting gastrin-releasing peptide receptors for cancer treatment. Anticancer Drugs. 2004; 15(10):921-7.
57. Spindel ER, Chin WW, Price J, Rees LH, Besser GM, Habener JF. Cloning and characterization of cDNA encoding human gastrin-releasing peptide. Proc. Natl. Acad. Sci. 1984; 81: 5699-5703.
58. Muller SZ, Gazdar AF, Minna JD. Molecular pathogenesis of lung cancer. Annu. Rev. Physiol. 2002; 64:681-708.
59. Flores DG, Lenz G, Roesler R, Schwartsmann G. Gastrin-releasing peptide receptor signalling in cancer. Cancer Therapy. 2009; 17: 331-345.
60. Richardson GE, Johnson BE. The biology of lung cancer. Semin. Oncol. 1993; 20:105–27
61. Cardona C, Rabbitts PH, Spindei ER, Ghatei MA, Bleehen NM, Bloom SR et al. Production of neuromedin B and neuromedin B gene expression in human lung tumor cell lines. Cancer Res. 1991; 51:5205–11
62. Cuttitta F, Carney DN, Mulshine J. Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature. 1985; 316:823-826.

63. Shriver SP, Bourdeau HA, Gubish CT. Sex-specific expression of gastrin-releasing peptide receptor: relationship to smoking history and risk of lung cancer. J Natl Cancer Inst. 2000; 92:24-33.

64. Halmos G, Schally AV. Reduction in receptors for bombesin and epidermal growth factor in xenografts of human small-cell lung cancer after treatment with bombesin antagonist RC-3095. Proc Natl Acad Sci USA. 1997; 94:956-960.

65. Waters CM, MacKinnon AC, Cummings J, Tufail-Hanif U, Jodrell D, Haslett C, Sethi T. Increased gastrin-releasing peptide (GRP) receptor expression in tumour cells confers sensitivity to [Arg6,D-Trp7,9,NmePhe8]-substance P (6-11)-induced growth inhibition. Br J Cancer. 2003; 88(11):1808-16.

66. Takahashi T, Obata Y, Sekido Y, Hida T, Ueda R, Watanabe H et al. Expression and amplification of myc gene family in small cell lung cancer and its relation to biological characteristics. Cancer Res. 1989; 49(10):2683-8.

67. Grandori C, Cowley SM, James LP, Eisenman RN. The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol. 2000; 16:653-99.

68. Funa K, Steinholtz L, Nou E, Bergh J. Increased expression of N-myc in human small cell lung cancer biopsies predicts lack of response to chemotherapy and poor prognosis. Am J Clin Pathol. 1987; 88(2):216-20.

69. Adachi J, Ookawa K, Shiseki M, Okazaki T, Tsuchida S, Morishita K et al. Induction of apoptosis but not G1 arrest by expression of the wild-type p53 gene in small cell lung carcinoma. Cell Growth Differ. 1996;7(7):879-86.

70. Jiang SX, Sato Y, Kuwao S, Kameya T. Expression of bcl-2 oncogene protein is prevalent in small cell lung carcinomas. Pathol. 1995; 177(2):135-8.

71. Ikegaki N, Katsumata M, Minna J, Tsujimoto Y. Expression of bcl-2 in small cell lung carcinoma cells. Cancer Res. 1994; 54(1):6-8.

72. Sidransky D, Hollstein M. Clinical implications of the p53 gene. Annu. Rev. Med. 1996; 47:285–301.

73. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2002; 2:489–501.

74. Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, Tsichlis PN et al. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev. 1997; 11(6):701-13.

75. Moore SM, Rintoul RC, Walker TR, Chilvers ER, Haslett C, Sethi T. The presence of a constitutively active phosphoinositide 3-kinase in small cell lung cancer cells mediates anchorage-independent proliferation via a protein kinase B and p70s6k-dependent pathway. Cancer Res. 1998; 58**:** 5239-47.

76. Walls M, Baxi SM, Mehta PP, Liu KK, Zhu J, Estrella H et al. Targeting small cell lung cancer harboring PIK3CA mutation with a selective oral PI3K inhibitor PF-4989216. Clin Cancer Res. 2014; 20(3):631-43.

77. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 2007; 6:273-286.

78. Ferrara N. VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer 2002; 2:795-803.

79. Hasegawa Y, Takanashi S, Okudera K, Kumagai M, Hayashi A, Morimoto T et al. Vascular endothelial growth factor level as a prognostic determinant of small cell lung cancer in Japanese patients. Intern Med. 2005; 44(1):26-34.

80. Litz J, Sakuntala Warshamana-Greene G, Sulanke G, Lipson KE, Krystal GW. The multi-targeted kinase inhibitor SU5416 inhibits small cell lung cancer growth and angiogenesis, in part by blocking Kit-mediated VEGF expression. Lung Cancer. 2004; 46(3):283-91.

81. Chen P, Zhu J, Liu DY, Li HY, Xu N, Hou M. Over-expression of survivin and VEGF in small-cell lung cancer may predict the poorer prognosis. Med Oncol 2014; 31(1):775.

82. Ueno K, Inoue Y, Kawaguchi T, Hosoe S, Kawahara M. Increased serum levels of basic fibroblast growth factor in lung cancer patients: relevance to response of therapy and prognosis. Lung Cancer. 2001; 31(2-3):213-9.

83. Rygaard K, Nakamura T, Spang-Thomsen M. Expression of the proto-oncogenes c-met and c-kit and their ligands, hepatocyte growth factor/scatter factor and stem cell factor, in SCLC cell lines and xenografts. Br J Cancer. 1993; 67(1):37-46.

84. Maulik G, Kijima T, Ma PC, Ghosh SK, Lin J, Shapiro GI, Schaefer E, Tibaldi E, Johnson BE, Salgia R. Modulation of the c-Met/hepatocyte growth factor pathway in small cell lung cancer. Clin Cancer Res. 2002; 8(2):620-7.

85. Bonomi P. Matrix metalloproteinases and matrix metalloproteinase inhibitors in lung cancer. Semin Oncol. 2002; 29:78-86.

86. Michael M, Babic B, Khokha R, Tsao M, Ho J, Pintille M et al. Expression and prognostic significance of metalloproteinases and their tissue inhibitors in patients with small-cell lung cancer. J Clin Oncol. 1999; 17:1802-8.

87. Li Z, Guo Y, Zhang H, Zhang T, Jin C, Young CY et al. Differential regulation of MMPs by E2F1, Sp1 and NF-kappa B controls the small cell lung cancer invasive phenotype. BMC Cancer. 2014; 14:276.

88. Burger M, Glodek A, Hartmann T, Schmitt-Graff A, Silberstein LE, Fujii N et al. Functional expression of CXCR4 (CD184) on small-cell lung cancer cells mediates migration, integrin activation, and adhesion to stromal cells. Oncogene. 2003; 22(50): 8093-101

89. Hartmann TN, Burger M, Burger JA. The role of adhesion molecules and chemokine receptor CXCR4 (CD184) in small cell lung cancer. J Biol Regul Homeost Agents. 2004; 18(2):126-30.

90. Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. J Cell Sci. 2012; 125, 5591-5596.

91. Ozbek S, Balasubramanian PG, Chiquet-Ehrismann R, Tucker RP, Adams JC. The evolution of extracellular matrix. Mol Biol Cell. 2010; 21(24): 4300-5.

92. Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol. 2012 Feb 20;196(4):395-406.

93. Fridman R, Giaccone G, Kanemoto T, Martin GR, Gazdar AF, Mulshine JL. Reconstituted basement membrane (matrigel) and laminin can enhance the tumorigenicity and the drug resistance of small cell lung cancer cell lines. Proc Natl Acad Sci U S A. 1990; 87:6698-702.

94. Sethi T, Rintoul RC, Moore SM, MacKinnon AC, Salter D, Choo C et al. Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. Nat Med. 1999; 5:662-8.

95. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechanoregulation of connective tissue remodeling. Nature Rev. Mol. Cell Biol. 2002; 3, 349–363.

96. Parsonage G, Filer AD, Haworth O, Nash GB, Rainger GE et al. A stromal address code defined by fibroblasts. Trends Immunol. 2005; 26, 150–156.

97. Li B, Wang JH. Fibroblasts and myofibroblasts in wound healing: force generation and measurement. J. Tissue Viability. 2011; 20, 108-120.

98. Sugimoto H, Mundel TM, Kieran MW, Kalluri R. Identification of fibroblast heterogeneity in the tumor microenvironment. Cancer Biol. Ther. 2006; 5, 1640-1646.

99. Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer. 2006; 6(5):392-401.

100. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005; 121, 335-348.

101. Langdon S, Sethi T, Ritchie A, Muir M, Smyth J, Rozengurt E. Broad spectrum neuropeptide antagonists inhibit the growth of small cell lung cancer *in vivo*. Cancer Res. 1992; 52(16): 4554-7.

102. Fischer JR, Darjes H, Lahm H, Schindel M, Drings P, Krammer PH. Constitutive secretion of bioactive transforming growth factor beta 1 by

small cell lung cancer cell lines. Eur J Cancer. 1994; 30:2125-9.

103. Rozengurt E, Sinnett-Smith J. Bombesin stimulation of DNA synthesis and cell division in cultures of Swiss 3T3 cells. Proc Natl Acad Sci U S A. 1983; 80:2936-40.

104. Eickelberg O, Kohler E, Reichenberger F, Bertschin S, Woodtli T, Erne P et al. Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3. Am J Physiol. 1999; 276:814-24.

105. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumorpromoting inflammation in an NF-kappaB-dependent manner. Cancer Cell. 2010; 17, 135-147.

106. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol. 2006;6:715-27.

107. Shiao SL, Ganesan AP, Rugo HS, Coussens LM. Immune microenvironments in solid tumors: new targets for therapy. Genes Dev. 2011; 15;25(24):2559-72.

108. Yu P, Fu YX. Tumor-infiltrating T lymphocytes: friends or foes? Lab Invest. 2006; 86(3):231-45.

109. Fridman WH, Page`s F, Saute`s-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat. Rev. Cancer. 2012; 12:298-306.

110. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J. Clin. Oncol.

2006; 24:5373-5380.

111. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat. Med. 2004; 10:942-949.
112. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin. Cancer Res. 2006; 12: 5423-5434.
113. Gomes AQ, Martins DS, Silva-Santos B. Targeting γδ T lymphocytes for cancer immunotherapy: from novel mechanistic insight to clinical application. Cancer Res. 2010; 70, 10024-10027.
114. Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stageovarian cancer. Cancer Res. 2001; 61:4766-72.
115. Hsieh CS, Lee HM, Lio CW. Selection of regulatory T cells in the thymus. Nat. Rev. Immunol. 2012; 12:157-167.
116. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J. Immunol.1995; 155, 1151-1164.
117. Seddon B., Mason D. Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. J. Exp. Med. 1999; 189, 877-882.
118. Woo EY, Yeh H, Chu CS, Schlienger K, Carrol RG, Riley JL et al. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit

autologous T cell proliferation. J Immunol. 2002; 168(9):4272-6.

119. Terabe M, Berzofsky JA. Immunoregulatory T cells in tumour Immunity. Curr Opin Immunol 2004; 16: 157.

120. Wei WZ, Morris GO, Kong YC. Anti-tumour immunity and autoimmunity: A balancing act of regulatory T cells. Cancer Immunol Immunother. 2004;53:73.

121. Brusko TM, Hulme MA, Myhr CB, Haller MJ, Atkinson MA. Assessing the in vitro suppressive capacity of regulatory T cells. Immunol Invest. 2007; 36:607-28.

122. Askenasy N., Kaminitz K., Yarkoni S. Mechanisms of T regulatory cell function. Autoimmun Rev. 2008; 7(5): 370-5.

123. Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T regulatory cells. Immunol. Rev. 2001; 182:68.

124. Weiner HL. Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells. Immunol. Rev. 2001; 182:207.

125. Kemper C, Chan AC, Green JM, Brett KA, Murphy KM, Atkinson JP. Activation of human $CD4^+$ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. Nature. 2003; 421:388.

126. Groux H. Type 1 T-regulatory cells: their role in the control of immune responses. Transplantation. 2003; 75:8S.

127. Ling KL, Pratap SE, Bates GJ, Singh B, Mortensen NJ, George BD et al. Increased frequency of regulatory T cells in peripheral blood and tumour infiltrating lymphocytes in colorectal cancer patients. Cancer Immun. 2007; 7:7.

128. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased

populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin Cancer Res. 2003; 9:4404-8.

129. Ormandy LA, Hillemann T, Wedemeyer H, Manns MP, Greten TF, Korangy F. Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. Cancer Res. 2005; 65:2457-64.
130. Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory Tcells in the peripheral blood of cancer patients. Clin Cancer Res 2003; 9:606-12.
131. McCarter MD, Baumgartner J, Escobar GA, Richter D, Lewis K, Robinson W et al. Immunosuppressive dendritic and regulatory T cells are upregulated in melanoma patients. Ann Surg Oncol 2007; 14:2854-60.
132. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. Nature Reviews Immunology. 2012; 12, 239-252.
133. Coca S, Perez-Pigueras J, Martinez D, Colmenarejo A, Saez MA, Vallejo C et al. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. 1997; Cancer 79, 2320–2328.
134. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Che X, Uwashige H et al. Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer. 2000; 88 (3):577-83.
135. Villegas FR, Coca S, Villarrubia VG, Jimenez R, Chillon MJ, Jareno J et al. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer. 2002; 35(1): 23-8.

136. Donskov F, Von der Maase H. Impact of immune parameters on long-term survival in metastatic renal cell carcinoma. J. Clin. Oncol. 2006; 24:1997–2005.

137. Zhu LY, Zhou J, Liu YZ, Pan WD. Prognostic significance of natural killer cell infiltration in hepatocellular carcinoma. Ai Zheng. 2009; 28:1198–1202.

138. Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H et al. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. Clin.Cancer Res. 2005; 11:7322-7327.

139. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat. Rev. Immunol. 2012; 12: 253-268.

140. Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. J. Clin. Invest. 2007; 117:1155-1166.

141. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol. 2003; 24:301-305.

142. Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J et al. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. Cancer Res. 2006; 66:1123-1131.

143. Sinha P, Clements VK, Bunt SK, Albelda SM, Ostrand-Rosenberg S. Cross-talk between myeloid-derived suppressor cells and Macrophages subverts tumor immunity toward a type 2 response. J.Immunol. 2007; 179:977-983.

144. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. Cancer Res. 2006; 15;66(2):605-12.

145. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010; 2;141(1):39-51.

146. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002; 23:549–555.

147. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. Immunity. 2014; 41:49–61.

148. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J. Pathol. 2002; 196, 254-265.

149. Ruffell B, Affara NI, Coussens LM. Differential macrophage programming in the tumour microenvironment. Trends Immunol. 2012; 33(3): 119-26.

150. Swiecki M, Colonna M. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. Immunol. Rev. 2010; 234:142–162.

151. Perrot I, Blanchard D, Freymond N, Isaac S, Guibert B, Pacheco Y et al. Dendritic cells infiltrating human non-small cell lung cancer are blocked at immature stage. J. Immunol. 2007; 178:2763–2769.

152. Bellone G, Carbone A, Smirne C, Scirelli T, Buffolino A, Novarino A et al. Cooperative induction of a tolerogenic dendritic cell phenotype by cytokines secreted by pancreatic carcinoma cells. J. Immunol. 2006; 177:3448–3460.

153. Lee BN, Follen M, Rodriquez G, Shen DY, Malpica A, Shearer WT et al.

Deficiencies in myeloid antigen-presenting cells in women with cervical squamous intraepithelial lesions. Cancer. 2006; 107(5): 999-1007.

154. Ormandy LA, Farber A, Cantz T, Petrykowska S, Wedemeyer H, Horning M et al. Direct ex vivo analysis of dendritic cells in patients with hepatocellular carcinoma. World J Gastroenterol. 2006; 12(20):3275-82.

155. Pinzon-Charry A, Maxwell T, Lopez JA. Dendritic cell dysfunction in cancer: a mechanism for immunosuppression. Immunol. Cell Biol. 2005; 83:451–461.

156. Pinzon-Charry A, Ho CS, Maxwell T, MuGuckin MA, Schmidt C, Furnival C et al. Numerical and functional defects of blood dendritic cells in early- and late-stage breast cancer. Br. J. Cancer 2007; 97:1251–1259.

157. Ghiringhelli F, Puig PE, Roux S, Parcellier A, Schmitt E, Solary E et al. Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. J Exp Med. 2005; 202(7): 919-29.

158. Dumitriu IE, Dunbar DR, Howie SE, Sethi T, Gregory CD. Human dendritic cells produce TGF-β1 under the influence of lung carcinoma cells and prime the differentiation of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells. J. Immunol. 2009; 182:2795–2807.

159. Lin A, Schildknecht A, Nguyen LT, Ohashi PS. Dendritic cells integrate signals from the tumor microenvironment to modulate immunity and tumor growth. Immunol. Lett. 2010; 127:77–84.

160. Johnston-Early A, Cohen MH, Fossieck BE Jr, Harwood S, Ihde DC, Bunn PA Jr et al. Delayed hypersensitivity skin testing as a prognostic indicator in patients with small cell lung cancer. Cancer. 1983; 52(8):1395-400.

161. Nakamura H, Saji H, Ogata A, Hosaka M, Hagiwara M, Kawasaki N et al. Immunologic parameters as significant prognostic factors in lung cancer. Lung Cancer. 2002; 37(2):161-9.

162. Masuno T, Ikeda T, Yokota S, Komuta K, Ogura T, Kishimoto S. Immunoregulatory T-lymphocyte functions in patients with small cell lung cancer. Cancer Res. 1986; 46:4195-9.

163. Fischer JR, Schindel M, Bülzebruck H, Lahm H, Krammer PH, Drings P. Decrease of interleukin-2 secretion is a new independent prognostic factor associated with poor survival in patients with small-cell lung cancer. Ann Oncol. 1997; 8:457-61.

164. Ioachim HL, Dorsett BH, Paluch E. The immune response at the tumor site in lung carcinoma. Cancer. 1976; 38:2296-2309.

165. Kurnick JT, Kradin RL, Blumberg R, Schneeberger EE, Boyle LA. Functional characterization of T lymphocytes propagated from human lung carcinomas. Clin Immunol Immunopathol. 1986; 38:367-80.

166. Pouniotis DS, Plebanski M, Apostolopoulos V, McDonald CF. Alveolar macrophage function is altered in patients with lung cancer. Clin Exp Immunol. 2006; 143:363-72.

167. Möller P, Hämmerling GJ. The role of surface HLA-A,B,C molecules in tumour immunity. Cancer Surv. 1992; 13:101-27.

168. Doyle A, Martin WJ, Funa K, Gazdar A, Garney D, Martin SE et al. Markedly decreased expression of class I histocompatibility antigens, protein, and mRNA in human small-cell lung cancer. J Exp Med. 1985; 161:1135-51.

169. Keir ME, Butte MJ, Freeman GJ and Sharpe AH. PD-1 and its ligands in

tolerance and immunity.. Annu Rev Immunol. 2008;26:677-704.

170. Zitvogel L, Kroemer G. Targeting PD-1/PD-L1 interactions for cancer immunocherapy. Oncoimmunology. 2012; 1(8): 1223-1225.

171. Ishii H, Azuma K, Kawahara A, Yamada K, Imamura Y, Tokito T et al. Significance of programmed cell death-ligand 1 expression and its association with survival in patients with small cell lung cancer. J Thorac Oncol. 2015; 10(3):426-30.

172. Manke HG, Aulenbacher P, Rüster S, Drings P. Inhibition of antibody synthesis and expression of T-cell membrane markers by serum factors of patients with small cell carcinoma of the lung. Anticancer Res. 1986; 6:869-73.

173. Ikeda T, Masuno T, Ogura T, Watanabe M, Shirasaka T, Hara H et al. Characterization and purification of an immunosuppressive factor produced by a small cell lung cancer cell line. Jpn J Cancer Res. 1991; 82:332-8.

174. Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H. The central role of CD4(+) T cells in the antitumor immune response. J Exp Med. 1998;188:2357-68.

175. Dougan M. Dranoff G. Immune therapy for cancer. Annu Rev Immunol. 2009; 27:83-117.

176. Nishikawa H, Sakaguchi S. Regulatory T cells in cancer immunotherapy. Curr Opin Immunol. 2014; 27:1-7.

177. Bremnes RM, Sundstrom S, Aasebø U, Kaasa S, Hatlevoll R, Aamdal S. Norweigian Lung Cancer Study Group . The value of prognostic factors in small cell lung cancer: results from a randomised multicenter study with

minimum 5 year follow-up. Lung Cancer. 2003; 39 (3): 303-313 .

178. Mahoney KM, Rennert PD, Freeman GJ. Combination cancer immunotherapy and new immunomodulatory targets. Nat Rev Drug Discov. 2015; 14(8): 561-84.
179. Pawelec G. Immunotherapy and immunoselection -- tumour escape as the final hurdle. FEBS Lett. 2004;567:63-6.
180. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011; 331(6024):1565-70.
181. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol. 1994; 12:991-1045.
182. Pardoll D. Does the immune system see tumours as foreign or self? Annu Rev Immunol. 2003; 21:807-39.
183. Bishara A, Malka R, Brautbar C, Barak V, Cohen I, Kedar E. Cytokine production in human mixed leukocyte reactions performed in serum-free media. J Immunol Methods. 1998;215:187-90.
184. Kennedy R. Celis E. Multiple roles for CD4+ T cells in anti-tumour immune responses. Immunol Rev. 2008; 222:129-44.
185. Chatila T, Silverman L, Miller R, Geha R. Mechanisms of T cell activation by calcium ionophore ionomycin. J Immunol. 1989; 143(4):1283-9.
186. Soltis RD, Hasz D, Morris MJ, Wilson ID. The effect of heat inactivation of serum on aggregation of immunoglobulins. Immunology. 1979; 36(1): 37-45.
187. Smith K.A. Interleukin-2: inception, impact and implications. Science. 1988; 240:1169.

188. Nelson B.H. IL-2, Regulatory T cells, and tolerance. J Immunol. 2004; 172(7): 3983-8.

189. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986; 136: 2348–2357.

190. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. Annu Rev Immunol. 2009; 27:485-517.

191. Gajewski TF, Meng Y, Blank C, Brown I, Kacha A, Kline J, Harlin H. Immune resistance orchestrated by the tumor microenvironment. Immunol Rev. 2006;213:131-45.

192. Wahl SM, Wen J, Moutsopoulos N. TGF-beta: a mobile purveyor of immune privilege. Immunol Rev. 2006;213:213-27.

193. Liu VC, Wong LY, Jang T, Shah AH, Park I, Yang X et al. Tumor evasion of the immune system by converting CD4+CD25- T cells into CD4+CD25+ T regulatory cells: role of tumor-derived TGF-beta. J Immunol. 2007;178:2883-92.

194. Levings MK, Bacchetta R, Schulz U, Roncarolo MG. The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. Int Arch Allergy Immunol. 2002;129:263-76.

195. Stephens LA, Mottet C, Mason D, Powrie F. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. Eur J Immunol. 2001;31:1247-54.

196. Rubbert A, Manger B, Lang N, Kalden JR, Platzer E. Functional characterization of tumor-infiltrating lymphocytes, lymph-node lymphocytes

and peripheral-blood lymphocytes from patients with breast cancer. Int J Cancer. 1991;49:25-31.

197. Guilloux Y, Viret C, Gervois N, Le Drean E, Pandolfino MC, Diez E et al. Defective lymphokine production by most CD8+ and CD4+ tumor-specific T cell clones derived from human melanoma-infiltrating lymphocytes in response to autologous tumor cells in vitro. Eur J Immunol. 1994;24:1966-73.
198. Kryczek I, Wei S, Zou L, Altuwaijri S, Szeliga W, Kolls J et al. Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. J Immunol. 2007; 178(11):6730-3.
199. Kryczek I, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S et al. Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. Blood. 2009; 114(6):1141-9.
200. Zou W, Restifo N. TH17 cells in tumour immunity and immunotherapy. Nat Rev Immunol. 2010; 10(4): 248-256.
201. Kryczek I, Wei S, Szeliga W, Vatan L and Zou W. Endogenous IL-17 contributes to reduced tumor growth and metastasis. Blood 2009;114:357-359.
202. Benchetrit F, Ciree A, Vives V, Warnier G, Gey A, Sautes-Fridman C et al. Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. Blood 2002;99:2114-2121.
203. Wang L, Yi T, Kortylewski M, Pardoll DM, Zeng D, Yu H. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. J Exp Med. 2009; 206(7):1457-64.

204. Nam JS, Terabe M, Kang MJ, Chae H, Voong N, Yang YA et al. Transforming growth factor beta subverts the immune system into directly promoting tumor growth through interleukin-17. Cancer Res 2008;68:3915-3923.

205. Muranski P, Restifo NP. Does IL-17 promote tumour growth? Blood. 2009;114:231–232.

206. Dennis KL, Blatner NR, Gounari F, Khazaie K. Current status of interleukin-10 and regulatory T cells. Curr Opin Oncol 2013; 25(6): 637-45.

207. Taylor A, Verhagen J, Blaser K, Akdis M, Akdis C. Mechanisms of immune suppression by interleukin-10 and transforming growth facor-β: the role of T regulatory cells. Immunology 2006; 117(4): 433-442.

208. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-β regulation of immune responses. Annu Rev Immunol. 2006: 24:99-146.

209. Taga K, Tosato G. IL-10 inhibits human T cell proliferation and IL-2 production. J Immunol. 1992; 148(4): 1143-8.

210. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R et al. Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T cell growth. J. Exp. Med. 1986; 163:1037.

211. Sakaguchi S, Miyara M, Costantino C, Hafler D. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol. 2010; 10(7):490-500.

212. Hsieh CS, Lee HM, Lio CW. Selection of regulatory T cells in the thymus. Nat. Rev. Immunol. 2012;12,157-167.

213. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol. 2004; 22: 531-62.

214. Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DAA. The development and function of regulatory T cells. Cell Mol Life Sci. 2009; 66(16): 2603–2622.

215. Woo EY, Chu CS, Goietz TJ, Shilenger K, Yeh H, Coukos G et al. Regulatory $CD4^{+}CD25^{+}$ T cells in tumours from patients with early- stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res. 2001: 61, 4766–4772.

216. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G et al. Prevalence of regulatory T cells is increased in peripheral blood and tumour microenvironment of patients with pancreas or breast adenocarcinoma. J. Immunol. 2002; 169, 2756–2761.

217. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H et al. Increased populations of regulatory T cells in peripheral blood and tumour-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin. Cancer Res. 2003; 9, 4404–4408.

218. Karube K, Ohshima K, Tsuchiya T, Tamaguchi T, Kawano R, Suzumiya J et al. Expression of FoxP3, a key molecule in CD4CD25 regulatory T cells, in adult T-cell leukaemia/lymphoma cells. Br. J. Haematol. 2004; 126, 81–84.

219. Viguier M, Lemaitre F, Verola O, Cho MS, Gorochov G, Dubertret L et al. Foxp3 expressing $CD4^{+}CD25^{high}$ regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of

infiltrating T cells. J. Immunol. 2004; 173, 1444–1453.

220. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol. 2006; 6(4):295-307.

221. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nature Med. 2004; 10, 942–949.

222. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity. 2009; 30(6):899-911.

223. Nishikawa H, Sakaguchi S. Regulatory T cells in tumour immunity. Int J Cancer. 2010; 127(4):759-67.

224. Seo N, Hayakawa S, Takigawa M, Tokura Y. Interleukin-10 expressed at early tumour sites induces subsequent generation of $CD4^+$ T-regulatory cells and systemic collapse of antitumour immunity. Immunology. 2001; 103, 449–457.

225. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N et al. Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-β induction of transcription factor Foxp3. J. Exp. Med. 2003; 198, 1875–1886.

226. Askenasy N, Ayelet Kaminitz A, Yarkoni S., Mechanisms of T regulatory cell function. Autoimmun Rev. 2008; 7(5):370-5.

227. Bacchetta R, Gregori S, Roncarolo MG. CD4+ regulatory T cells: mechanisms of induction and effector function. Autoimmun Rev. 2005; 4(8): 491-6.

228. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay

A et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 2006; 203:1693–700.

229. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4. T reg cells. J Exp Med 2006;203:1701–11.
230. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, Shevach EM. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol. 2010; 184(7):3433-41.
231. Akimova T, Beier UH, Wang L, Levine MH, Hancock WW. Helios expression is a marker of T cell activation and proliferation. PLoS One. 2011; 6(8):e24226.
232. Serre K, Benezech C, Desanti G, Bobat S, Toellner KM, Bird R et al. Helios is associated with CD4 T cells differentiating to T helper 2 and follicular helper T cells in vivo independently of Foxp3 expression. PLoS One. 2011; 6(6):e20731.
233. Gottschalk RA, Corse E, Allison JP. Expression of Helios in peripherally induced Foxp3+ regulatory T cells. J Immunol. 2012; 188(3):976-80.
234. Yadav M, Louvet C, Davini D, Gardner JM, Martinez-Llordella M, Balley-Bucktrout S et al. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. J Exp Med. 2012; 209(10):1713-22, S1-19.
235. Imamichi H, Sereti I, Lane HC. IL-15 acts as a potent inducer of CD4+CD25hi cells expressing FOXP3. Eur J Immunol 2008; 38:1621-30.

236. Xia J, Liu W, Hu B, Tian Z, Yang Y. IL-15 promotes regulatory T cell function and protects against diabetes development in NK-depleted NOD mice. Clin Immunol 2010; 134:130-9.

237. Thornton AM. Shevach EM. CD4+CD25+ immunoregularory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med. 1998; 188(2):287-96.

238. Carbone F, De Rosa V, Carrieri PB, Montella S, Bruzzese D, Porcellini A et al. Regulatory T cell proliferative potential is impaired in human autoimmune disease. Nat. Med. 2014; 20(1):69-74.

239. Vignali DA, Collison LW, Workman CJ. How regulatory T cell work. Nat Rev Immunol. 2008; 8(7): 523-32.

240. Bin Dhuban K, Kornete M, S Mason E, Piccirillo CA. Functional dynamics of Foxp3^{+} regulatory T cells in mice and humans. Immunol Rev. 2014; 259(1): 140-58.

241. Levings MK, Sangregorio R. Roncarolo MG. Human CD25+CD4+ T regulatory cells suppress naïve and memory T cell proliferation and can be expandedin vitro without loss of function. J. Exp. Med. 2001; 193:1295–1302.

242. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4+CD25+ T cells with regulatory properties isolated from peripheral blood. J. Exp. Med. 2001;1 93:1285–1294.

243. Baecher-Allan C, Brown JA, Freeman, GJ, Hafler, DA. CD4+CD25high regulatory cells in human peripheral blood. J. Immunol. 2001; 167, 1245–1253.

244. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. J Exp Med. 2001; 193(11):1303-10.

245. de la Rosa M, Rutz S, Dorninger H, Scheffold A. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. Eur J Immunol. 2004; 34(9):2480-8.

246. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. Nat Immunol. 2007; 8(12):1353-62.

247. Tran DQ et al. Analysis of adhesion molecules, target cells, and role of IL-2 in human FOXP3+ regulatory T cell suppressor function. J Immunol. 2009; 182(5):2929-38.

248. Vercoulen Y, Wehrens EJ, van Teijiingen NH, de Jager W, Beekman JM, Prakken BJ. Human regulatory T cell suppressive function is independent of apoptosis induction in activated effector T cells. PLoS One. 2009; 4(9):e7183.

249. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med. 2001; 194(5):629-44.

250. Levings MK, Sangregorio R, Sartirana C, Moschin AL, Battaglia M, Orban PC et al. Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct

from type 1 T regulatory cells. J Exp Med. 2002; 196(10):1335-46.

251. Piccirillo CA, Letterio JJ, Thornton AM, MuHugh RS, Mamura M, Mizuhara H et al. CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. J Exp Med. 2002; 196(2):237-46.

252. Oberle N, Eberhardt N, Falk CS, Krammer PH, Suri-Payer E. Rapid suppression of cytokine transcription in human CD4+CD25 T cells by CD4+Foxp3+ regulatory T cells: independence of IL-2 consumption, TGF-beta, and various inhibitors of TCR signaling. J Immunol. 2007; 179(6):3578-87.

253. Mural M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat. Immunol. 2009; 10(11):1178-84.

254. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat Rev Immunol. 2006: 6(8): 595-601.

255. Muro S, Taha R, Tsicopoulos A, Olivenstein R, Tonnel AB, Christodoulopoulos P et al. Expression of IL-15 in inflammatory pulmonary diseases. J Allergy Clin Immunol. 2001; 108(6): 970-5.

256. Meazza R et al. Identification of a novel IL-15 transcript isoform generated by alternative splicing in human small cell lung cancer cell lines. Oncogene. 1996; 12(10): 2187-92.

257. Lin SJ, Lu CH, Yan DC, Lee PT, Hsiao HS, Kuo ML. Expansion of regulatory T cells from umbilical cord blood and adult peripheral blood

$CD4^+CD25^+$ T cells. Immunol. Res. 2014; 60: 105–111.

258. Raynor J, Sholl A, Plas DR, Bouillet P, Chougnet CA, Hildeman DA. IL-15 fosters age-driven regulatory T cell accrual in the face of declining IL-2 levels. Front. Immunol. 2013; 4: 161.

259. Marshall D, Sinclair C, Tung S, Seddon B. Differential requirement for IL-2 and IL-15 during bifurcated development of thymic regulatory T cells. J. Immunol. 2014;193: 5525–5533.

260. Litjens NH, Boer K, Zuijderwijk JM, Klepper M, Peeters AM, Prens EP et al. Allogeneic mature human dendritic cells generate superior alloreactive regulatory T cells in the presence of IL-15. J. Immunol. 2015; 194:5282–5293.

261. Patel O, Shulkes A, Baldwin GS. Gastrin-releasing peptide and cancer. Biochim Biophys Acta. 2006; 1766(1):23-41.

262. Vangsted AJ, Schwartz TW. Production of gastrin-releasing peptide- (18–27) and a stable fragment of its precursor in small cell lung carcinoma cells, J. Clin. Endocrinol. Metab. 1990; 70:1586–1593.

263. Holst JJ, Hansen M, Bork E, Schwartz TW. Elevated plasma concentrations of C-flanking gastrin-releasing peptide in small-cell lung cancer. J. Clin. Oncol.1989; 7:1831–1838.

264. Corjay MH, Dobrzanski DJ, Way JM, Viallet J, Shapira H, Worland P et al. Two distinct bombesin receptor subtypes are expressed and functional in human lung carcinoma cells. J Biol Chem. 1991; 266:18771-9.

265. Toi-Scott M, Jones CLA, Kane MA. Clinical correlates of bombesin-like peptide receptor subtype expression in human lung cancer cells. Lung Cancer 1996; 15:341-54.

266. Xiao D, Wang J, Hampton LL, Weber HC. The human gastrin-releasing peptide receptor gene structure, its tissue expression and promoter. Gene 2001; 264:95-103.

267. Cornelio DB, Roesier R, Schwartsmann G. Gastrin-releasing peptide receptor as a molecular target in experimental anticancer therapy. Ann Oncol. 2007; 18:1457-1466.

268. Avis IL, Kovacs TO, Kasprzyk PG, Treston AM, Batholomew R, Walsh JH et al. Preclinical evaluation of an anti-autocrine growth factor monoclonal antibody for treatment of patients with small cell lung cancer. J Natl Cancer Inst 1991; 83:1470–1476.

269. Fink R, Ehrhardt R, Dancygier H. Bombesin and its analogues inhibit interleukin-2-induced proliferation of CTLL-2 cells. Regul Pept. 1988;23:323-30.

270. Del Rio M, Hernanz A, de la Fuente M. Bombesin, gastrin-releasing peptide, and neuromedin C modulate murine lymphocyte proliferation through adherent accessory cells and activate protein kinase C. Peptides. 1994;15:15-22.

271. Makarenkova VP, Shurin GV, Tourkova IL, Balkir L, Pirtskhalaishvili G, Perez L et al. Lung cancer-derived bombesin-like peptides down-regulate the generation and function of human dendritic cells. J. Neuroimmunol. 2003; 145: 55–67.

272. Ruff M, Schiffmann E, Terranova V, Pert CB. Neuropeptides are chemoattractants for human tumor cells and monocytes: a possible mechanism for metastasis. Clin. Immunol. Immunopathol. 1985; 37: 387–39.

273. Meloni F, Saporiti A, Ballabio P, Brocchieri A, Grignani G, Gialdroni Grassi G. In vitro effect of bombesin-related peptides on the procoagulant activity of alveolar macrophages. Monaldi Arch. Chest Dis. 1995; 50: 187–190.

274. Meloni F, Ballabio P, Bianchi L, Mangiarotti P, Grassi G, Bignamini A et al. Bombesin enhances monocyte and macrophage activities: possible role in the modulation of local pulmonary defenses in chronic bronchitis. Respiration. 1996; 63: 28–34.

275. Roy N, Pollard JW. Tumour-associated macrophages: from mechanisms to therapy. Immunity. 2014; 41(1): 49-61.

276. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013; 496:445-55.

277. Balkwill FR, Mantovani A. Cancer-related inflammation: common themes and therapeutic opportunities. Semin. Cancer Biol.2012; 22:33–40.

278. Biswas SK. Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol. 2010; 11(10): 889-96.

279. Sica A, BronteV. Altered macrophage differentiation and immune dysfunctionin tumor development. J. Clin. Invest. 2007; 117, 1155–1166.

280. Klimp AH, Hollema H, Kempinga C, van der Zee AG, de Vries EG, Daemen T. Expression of cyclooxygenase-2 and inducible nitric oxide synthase in human ovarian tumors and tumor-associated macrophages. Cancer Res 2001;61:7305–9.

281. Sica A, Saccani A, Bottazzi B, Polentarutti N, Vecchi A, van Damme J et al. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. J Immunol

2000;164:762–7.

282. Redente EF, Dwyer-Nield LD, Merrick DT, Raina K, Agarwal R, Pao W et al. Tumour progression stage and anatomical site regulate tumour-associated macrophage and bone marrow-derived monocyte polarization. Am J Pathol. 2010; 176:2972-2985.
283. Coussens LM, Zitvogel L, Palicka AK. Neutralizing tumour-promoting chronic inflammation: a magic bullet? Science. 2013; 339:286-291.
284. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase (PI3K) pathway in cancer. Nat Rev Drug Discov. 2009; 8(8): 627-644.
285. Mantovani A. Sica A. Sozzani S. Allavena P. Vecchi A. Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trens Immunol. 2004; 25(12):677-86.
286. Roszer T. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. Mediators Inflamm. 2015; 2015:816460.
287. Yang HK, Scott FM, Trepel JB, Battey JF, Johnson BE, Kelley MJ. Correlation of expression of bombesin-like peptides and receptors with growth inhibition by an anti-bombesin antibody in small-cell lung cancer cell lines. Lung Cancer. 1998; 21:165–175.
288. Toi-Scott M, Jones CLA, Kane MA. Clinical correlates of bombesin-like peptide receptor subtype expression in human lung cancer cells. Lung Cancer 1996; 15:341–354.
289. Shriver SP, Bourdeau HA, Gubish CT, Tirpak DL, Davis AL, Luketich JD et al. Sex-specific expression of gastrin-releasing peptide receptor:

relationship to smoking history and risk of lung cancer. J Nati Cancer Inst. 2000; 92(1):24-33.

290. Egloff AM, Gaither Davis A, Shuai Y, Land S, Pilewski JM, Luketich JD et al. Gastrin-releasing peptide receptor expression in non-cancerous bronchial epithelia is associated with lung cancer: a case-control study. Respir Res. 2012; 13:9.

291. Uchida K, Kojima A, Morokawa N, Tanabe O, Anzai C, Kawakami M et al. Expression of progastrin-releasing peptide and gastrin-releasing peptide receptor mRNA transcripts in tumor cells of patients with small cell lung cancer. J Cancer Res Clin Oncol 2002; 128:633–640.

292. Mattei J, Achcar RD, Cano CH, Macedo BR, Meurer L, Batlle BS et al. Gastrin-releasing peptide receptor expression in lung cancer. Arch Pathol Lab Med. 2014; 138(1):98-104.

293. Alexander RW, Upp JR Jr, Poston GJ, Gupta V, Townsend CM Jr, Thompson JC. Effects of bombesin on growth of human small cell lung carcinoma in vivo. Cancer Res. 1988; 48:1439–1441.

294. Koppan M, Halmos G, Arencibia JM, Lamharzi N, Schally AV. Bombesin/gastrin-releasing peptide antagonists RC-3095 and RC-3940-II inhibit tumor growth and decrease the levels and mRNA expression of epidermal growth factor receptors in H-69 small cell lung carcinoma, Cancer 1998; 83:1335–1343.

295. Thomas F, Arvelo F, Antoine E, Jacrot M, Poupon MF. Antitumoral activity of bombesin analogues on small cell lung cancer xenografts: relationship with bombesin receptor expression, Cancer Res. 1992; 52: 4872–4877.

296. Mahmoud S, Staley J, Taylor J, Bogden A, Moreau JP, Coy D et al. [Psi

13,14] bombesin analogues inhibit growth of small cell lung cancerin vitro and in vivo. Cancer Res. 1991; 51:1798–1802.

297. Kiaris H, Schally AV, Nagy A, Sun B, Armatis P, Szepeshazi K. Targeted cytotoxic analogue of bombesin/gastrin-releasing peptide inhibits the growth of H-69 human small-cell lung carcinoma in nude mice, Br. J. Cancer. 1999; 81: 966–971.

298. Layton JE, Scanlon DB, Soveny C, Morstyn G. Effects of bombesin antagonists on the growth of small cell lung cancer cells in vitro, Cancer Res. 1988; 48:4783–4789.

299. Dunzendorfer S, Wiedermann CJ. Neuropeptides and the immune system: focus on dendritic cell. Crit. Rev. Immunol. 2001; 21:523-557.

300. Elitsur Y, Freedland CP, Luk GD. Inhibition of DNA synthesis in human peripheral and lamina propria lymphocytes by 5-aminosalicylic acid and hydrocortisone. Reg. Immunol. 1990; 3:56-61.

301. Zhou S, Potts EN, Cuttitta F, Foster WM, Sunday ME. Gastrin-releasing peptide blockade as a broad-spectrum anti-inflammatory therapy for asthma. Proc Natl Acad Sci USA. 2011;108:2100–2105.

302. Petronilho F, Roesler R, Schwartsmann G, Dal Pizzol F. Gastrin-releasing peptide receptor as a molecular target for inflammatory diseases. Inflamm Allergy Drug Targets. 2007; 6:197–200.

303. Oliveira PG, Brenol CV, Edelweiss MI, Brenol JC, Petronilho F, Roesier R et al. Effects of an antagonist of the bombesin/gastrin-releasing peptide receptor on complete Freund's adjuvant-induced arthritis in rats. Peptides. 2008; 29:1726–1731.

304. Dal-Pizzol F, Di Leone LP, Ritter C, Martins MR, Reinke A, Pens Gelain D

et al. Gastrin-releasing peptide receptor antagonist effects on an animal model of sepsis. Am J Respir Crit Care Med. 2006; 173:84–90.

305. Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory microenvironment in tumor progression: the role of tumor-associated macrophages. Crit Rev Oncol Hematol. 2008;66:1-9.
306. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumour microenvironment. Nat. Immunol. 2013; 14:1014-1022.
307. Gordon S. Alternative activation of macrophages. Nat. Rev. Immunol. 2003; 3(1): 23-25.
308. Zizzo G, Hilliard BA, Monestier M, Cohen PL. Efficient clearance of early apoptotic cells by human macrophages requires M2c polarization and MerTK induction. J Immunol. 2012; 189(7): 3508-20.
309. Svensson-Arvelund J, Mehta RB, Lindau R, Mirrasekhian E, Rodriguez-Martinez H, Berg G et al. The Human Fetal Placenta Promotes Tolerance against the Semiallogeneic Fetus by Inducing Regulatory T Cells and Homeostatic M2 Macrophages. J Immunol. 2015; 194(4): 1534-44.
310. Bepler G, Zeymer U, Mahmound S, Fiskum G, Palaszynski E, Rotsch M et al. Substance P analogues function as bombesin receptor antagonists and inhibit small cell lung cancer clonal growth. Peptides. 1988; 9(6): 1367-72.
311. Jiang MH, Chung E, Chi GF, Ahn W, Lim JE, Hong HS et al. Substance P induces M2- type macrophages after spinal cord injury. Neuroreport. 2012; 23(13):786-92.
312. Tuluc F, Meshki J, Spitsin S, Douglas SD. HIV infection of macrophages is enhanced in the presence of increased expression of CD163 induced by substance P. J Leukoc Biol. 2014; 96(1): 143-50.

313. Iwamoto M, Shinohara H, Miyamoto A, Okuzawa M, Mabuchi H, Nohara T et al. Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. Int J Cancer. 2003;104:92-7.

314. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A. 2005;102:18538-43.

315. Taylor RC, Patel A, Panageas KS, Busam KJ, Brady MS. Tumor-infiltrating lymphocytes predict sentinel lymph node positivity in patients with cutaneous melanoma. J Clin Oncol. 2007;25:869-75.

316. Kärjä V, Aaltomaa S, Lipponen P, Isotalo T, Talja M, Mokka R. Tumour-infiltrating lymphocytes: A prognostic factor of PSA-free survival in patients with local prostate carcinoma treated by radical prostatectomy. Anticancer Res. 2005;25:4435-8.

317. Tsutsui S, Yasuda K, Suzuki K, Tahara K, Higashi H, Era S. Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. Oncol Rep. 2005;14:425-31.

318. Shiao SL, Ganesan AP, Rugo HS, Coussens LM. Immune microenvironments in solid tumours: new targets for therapy. Genes Dev. 2011; 25(24):2559-72.

319. Mougiakakos D, Johansson CC, Trocme E, All-Ericsson C, Economou MA, Larsson O et al. Intratumoral forkhead box P3-positive regulatory T cells predict poor survival in cyclooxygenase-2-positive uveal melanoma. Cancer 2010;116:2224–33.

320. Shen Z, Zhou S, Wang Y, Li RL, Zhong C, Liang C et al. Higher intratumoral infiltrated Foxp3. Treg numbers and Foxp3./CD8. ratio are associated with adverse prognosis in resectable gastric cancer. J Cancer Res Clin Oncol 2010;136:1585–95.

321. Alvaro T, Lejeune M, Salvado MT, Bosch R, Garcia JF, Jaen J et al. Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res. 2005; 11: 1467-1473.

322. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay Nei H et al. Prognostic value of tumor-infiltrating $CD4^+$ T-cell subpopulations in head and neck cancers. Clin Cancer Res. 2006; 12:465-472.

323. Winerdal ME, Marits P, Winerdal M, Hasan M, Rosenblatt R, Tolf A et al. FOXP3 and survival in urinary bladder cancer. BJU Int 2011;108:1672–8.

324. Lee WS, Park S, Lee WY, Yun SH, Chun HK. Clinical impact of tumor-infiltrating lymphocytes for survival in stage II colon cancer. Cancer 2010;116:5188–99.

325. Ladanyi A, Mohos A, Somiai B, Liszkay G, Gilde K, Fejos Z et al. FOXP3+ cell density in primary tumor has no prognostic impact in patients with cutaneous malignant melanoma. Pathol Oncol Res. 2010; 16(3): 303-9.

326. Sorrentino C, Musiani P, Pompa P, Cipollone G, Di Cario E. Androgen deprivation boosts prostatic infiltration of cytotoxic and regulatory T lymphocytes and has no effect on disease-free survival in prostate cancer patients. Clin Cancer Res. 2011; 17(6):1571-81.

327. Johnston-Early A, Cohen MH, Fossieck BE Jr, Harwood S, Ihde DC, Bunn PA Jr et al. Delayed hypersensitivity skin testing as a prognostic indicator

in patients with small cell lung cancer. Cancer. 1983; 52(8):1395 -1400.

328. Fischer JR, Schindel M, Stein N, Lahm H, Gallati H, Krammer PH et al. Selective suppression of cytokine secretion in patients with small-cell lung cancer. Ann Oncol.1995; 6(9):921-926 .

329. Fischer JR, Schindel M, Bülzebruck H, Lahm H, Krammer PH, Drings P. Decrease of interleukin-2 secretion is a new independent prognostic factor associated with poor survival in patients with small-cell lung cancer. Ann Oncol.1997; 8(5): 457-461.

330. Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. Regulatory T cells in cancer. Adv. Cancer Res. 2010; 107:57-117.

331. Eerola AK, Soini Y, Pääkkö P. A high number of tumor-infiltrating lymphocytes are associated with a small tumor size, low tumor stage, and a favorable prognosis in operated small cell lung carcinoma. Clin Cancer Res. 2000;6:1875-81.

332. Ugolini C, Basolo F, Proietti A, Vitti P, Elisei R, Miccoli P et al. Lymphocyte and immature dendritic cell infi ltrates in differentiated, poorly differentiated, and undifferentiated thyroid carcinoma. Thyroid. 2007; 17(5):389-393.

333. El-Gohary YM, Metwally G, Saad RS, Robinson MJ, Mesko T, Poppiti RJ. Prognostic significance of intratumoral and peritumoral lymphatic density and blood vessel density in invasive breast carcinomas. Am J Clin Pathol. 2008;129(4):578-86.

334. Hori S, Sakaguchi S. Foxp3: a critical regulator of the development and function of regulatory T cells. Microbes Infect. 2004; 6(8):745-51.

335. Karanikas V, Speletas M, Zamanakou M, Kalala F, Louies G, Kerenidi T et

al. FoxP3 expression in human cancer cells. J Transl Med. 2008; 6:19.

336. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin Cancer Res. 2006; 12:5423–5434.

337. Knutson KL, Disis ML, Salazar LG. CD4 regulatory T cells in human cancer pathogenesis. Cancer Immunol Immunother. 2007; 56(3):271-185.

338. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. Immunity. 2009; 30:616-625.

339. Munn DH. Blocking IDO activity to enhance anti-tumor immunity. Front Biosci. 2012; 4:734-45.

340. Johnson BF. Clay TM, Hobeika AC, Luerly HK, Morse MA. Vascular endothelial growth factor and immunosuppression in cancer: current knowledge and potential for new therapy. Expert Opin Biol Ther. 2007; 7(4):449-60.

341. Kumar V. Adenosine as an endogenous immunoregulator in cancer pathogenesis: where to go? Purinergic Signal. 2013; 9(2):145-65.

342. Liu FT, Rabinovich GA. Galectins as modulators of tumour progression. Nat Rev Cancer. 2005; 5(1):29-41.

343. Jakobisiak M, Golab J, Lasek W. Interleukin 15 as a promising candidate for tumor immunotherapy. Cytokine Growth Factor Rev. 2011;22:99–108.

344. Pagliari D, Frosali S, Landolfi R, Cianci R. The role of IL-15 in human cancer: Friend or Foe? International Trends in Immunity. 2013; 1:35-42.

345. Steel JC, Waldmann TA, Morris JC: Interleukin-15 biology and its therapeutic implications in cancer, Trends Pharmacol Sci. 2012; 33:35-41.

346. Malamut G, El Machhour R, Montcuguet N, Martin-Lanneree S, Dusanter-Fourt I, Verkarre V et al. IL-15 triggers an antiapoptotic pathway in human intraepithelial lymphocytes that is a potential new target in celiac disease-associated inflammation and lymphomagenesis, J Clin Invest, 2010; 120: 2131-43.

347. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature. 2007; 450(7169):566-9.

348. Bardel E, Larousserie F, Charlot-Rabiega P, Coulomb-L'Herminé A, Devergne O. Human CD4+ CD25+ Foxp3+ regulatory T cells do not constitutively express IL-35. J Immunol. 2008; 181(10):6898-905.

349. Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J et al. IL-35-mediated induction of a potent regulatory T cell population. Nat Immunol. 2010; 11(12):1093-101.

350. Chaturvedi V, Collison LW, Guy CS, Workman CJ, Vignali DA. Cutting edge: Human regulatory T cells require IL-35 to mediate suppression and infectious tolerance. J Immunol. 2011; 186(12):6661-6.

351. Lord SJ, Rajotte RV, Korbutt GS, Bleackley RC. Granzyme B: a natural born killer. Immunol Rev. 2003; 193:31-8.

352. Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP, Ley TJ. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. Blood. 2004; 104, 2840–2848.

353. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity. 2004; 21(4):589-601.

354. Cao X, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR et al. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. Immunity. 2007; 27(4): 635-46.

355. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. J Immunol. 2005; 174(4):1783-6.

356. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008; 454(7203):436-44.

357. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG et al. Macrophage polarization in tumour progression. Semin. Cancer. Biol. 2008; 18:349-355.

358. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008;8:958–969.

359. Ma J, Liu L, Che G, Yu N, Dai F, You Z. The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. BMC Cancer. 2010; 10:112.

360. Liu L, Ge D, Ma L, Mei J, Liu S, Zhang Q et al. Interleukin-17 and prostaglandin E2 are involved in formation of an M2 macrophage dominant microenvironment in lung cancer. J Thorac Oncol. 2012; 2:1091-100.

361. Quatromoni JG, Eruslanov E. Tumour-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. Am J Transl Res. 2012; 4(4):376-389.

362. Bremnes RM, Al-Shibli K, Donnem T, Sirera R, Al-Saad S, Andersen S et al. The Role of Tumor-Infiltrating Immune Cells and Chronic Inflammation at the Tumor Site on Cancer Development, Progression, and Prognosis: Emphasis on Non-small Cell Lung Cancer. J Thorac Oncol. 2011; 6(4): 824-33.

Chapter 9: Publications

CHEST

Original Research

LUNG CANCER

Histologic Assessment of Tumor-Associated CD45+ Cell Numbers Is an Independent Predictor of Prognosis in Small Cell Lung Cancer

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Background: **Small cell lung carcinoma (SCLC) continues to have a poor prognosis, with a 2-year survival of < 20%. Studies have suggested that SCLC may affect the immune system to allow it to evade immunologic responses. We hypothesized that any such effect would be characterized by a decrease in the lymphoid cells associated with the tumor in biopsy specimens and that this might relate to patient outcome.**

Methods: **Sixty-four SCLC biopsy specimens were immunohistochemically stained with anti-CD45 antibody to identify immune cells associated with the tumor. A mean CD45 count per high-power field for each case was obtained, and the results were correlated with age, sex, stage, performance status (PS), treatment with chemotherapy/radiotherapy, and overall survival.**

Results: **The median CD45 count for all cases was taken as 40 ($CD45_{40}$). Kaplan-Meier plots demonstrated better survival for patients with a $CD45_{40} > 40$ ($P < .009$). No relationship between $CD45_{40}$ and age, sex, stage, or treatment by chemotherapy or radiotherapy was identified. Although PS was a significant predictor of survival ($P = .014$), it did not correlate with $CD45_{40}$. In patients with better Eastern Cooperative Oncology Group PS (≤ 2), the $CD45_{40}$ demonstrated a highly significant survival advantage for those with $CD45_{40} > 40$ ($P < .0001$).**

Conclusions: **The data indicate that (1) simple immunohistochemical assessment of immune cell infiltrates in routinely processed and stained biopsy specimens of primary tumors can provide prognostic information in SCLC and (2) tumor-associated CD45+ cells in SCLC biopsy specimens may be a good clinical marker to identify patients with poor prognosis despite good PS.**

CHEST 2013; 143(1):146–151

Abbreviations: hpf = high-power field; PS = performance status; SCLC = small cell lung carcinoma

Lung cancer accounts for > 30,000 deaths per year in the United Kingdom.[1,2] Small cell lung carcinoma (SCLC) accounts for around 15% of all lung cancers and is characterized by rapid growth and early metastatic spread.[3] The tumor is often initially responsive to chemotherapy but frequently relapses because of the microscopic survival of chemoresistant cancer cells.[4] Prognosis is very poor, with a 2-year survival rate, despite treatment, of < 20%.[4] This figure has improved only marginally in the past 2 decades despite combination chemo- and radiotherapy regimens.[5] Currently, only a small number of indicators can be used to stratify patients with SCLC into prognostic groups. These include clinical markers such as performance status (PS) measured using the Eastern Cooperative Oncology Group scale (0-4), disease stage (limited or extensive), age, and sex.[6] Peripheral blood lactate dehydrogenase levels have also been shown to predict survival.[6] Defining new prognostic markers will assist medical decision making and improve future research design and may highlight potential therapeutic targets.

The host immune response to SCLC may play an important role in controlling tumor growth.[7] Infrequently, SCLC can present with paraneoplastic encephalomyelitis and peripheral neuropathy prior to clinical detection of the lung tumor.[8] In these cases, antibodies that react to proteins expressed on both SCLC and neurons (HuD, HuC, and Hel-N1) are frequently

detected in the patient's serum.[9] The early presentation of SCLC in these patients and the more favorable outcome from SCLC associated with paraneoplastic encephalomyelitis has led to the suggestion that these patients have developed an efficient antitumor immune response.[10] Thus, investigating the immune response to SCLC may reveal new prognostic markers.

Prior research has demonstrated several immune parameters that predict patient prognosis and disease stage in SCLC. These include the degree of delayed-type hypersensitivity reaction and defects in peripheral blood lymphocyte numbers, proliferative response, and secretion of the cytokine IL-2.[11-14] However, these studies have focused on the role of systemic immune dysfunction, which may relate to tumor burden and the patient's nutritional state. Furthermore, assessment of systemic immune defects relies on complicated tests of immunologic function and may not reflect the relevant changes in the local tumor environment. Studies in other cancers have demonstrated that tumor-infiltrating T lymphocytes and dendritic cells, which play a crucial role in the generation of tumor-specific effector T cells, are associated with improved patient survival and reduced relapse rate.[15-18] These data indicate that studies of the in situ antitumor immune response can define new prognostic markers. With this in mind, and conscious that any new marker should be simple and reproducible under clinical laboratory conditions, we sought to determine whether the presence of immune cells infiltrating primary lung tumors could be used as an accurate predictor of survival in SCLC.

Materials and Methods

SCLC Cases

Ethical and institutional management approval for the study was obtained (LREC/2004/8/16). Sixty-four biopsy samples with primary lung SCLC from the years 1999 to 2001, for which sufficient residual material was available for study and for which clinical data were available, were identified from the pathology archives at the Royal Infirmary of Edinburgh (Table 1). The cases selected included four resection specimens, 14 core biopsies, 43 bronchial biopsies, and three mediastinal biopsies. Fine-needle aspirate specimens of lung lesions and biopsy material from other tumor sites, including metastases, were not included. The histologic diagnosis of SCLC was reconfirmed in each case by assessment of the tumor morphology. Information regarding demographics (age and sex), tumor stage, treatment, Eastern Cooperative Oncology Group PS, and survival (days) was obtained from patient records.

Immunohistochemistry

Immunohistochemical staining for CD45$^+$ cells in the biopsy specimens was performed in the Department of Pathology, Royal Infirmary of Edinburgh, using antibodies to CD45 (clone 2B11 and PD7/26, Dako) in a BOND-MAX automated immunohistochemical staining machine (Leica Microsystems GmbH) according to the laboratory's standard protocol, including antigen retrieval with heat and citrate buffer.

Assessment of CD45 Infiltrate

Stained slides were viewed with a Leica microscope. The average number of CD45$^+$ cells per high-power field (hpf) was determined for each SCLC tumor sample. CD45$^+$ cells were counted by three independent observers using light microscopy from at least six randomly selected × 400 hpfs/tumor sample. The area of the field at this magnification was 0.18 mm^2. Fields were not counted if they did not contain tumor cells.

Statistical Analysis

A level of ± 40 CD45$^+$ cells/hpf was used to define the population in terms of levels of CD45 ($CD45_{40}$: $CD45 \leq 40$, $n = 34$; $CD45 > 40$, $n = 30$). Analysis of variance and the χ^2 test for association and for trend were used to examine the association between CD45 and other possible predictors of survival. Univariate analysis of survival times was performed using the Kaplan-Meier methods of estimation, with the log rank (Mantel-Cox) test for significance (null hypothesis: the distribution of survival times is equal for each level of $CD45_{40}$). The Cox proportional hazards model was used to examine multiple variables in one model.

Results

CD45 Counts and Overall Survival

Immunohistochemical staining of the sections highlighted the presence of CD45$^+$ cells within the sections examined (Fig 1). The CD45 counts obtained for the 64 cases had an average of 37.6 positive cells per field, with a range of 2.8 to 127.4. The range and average value for each case counted is shown in Figure 2. Analysis of interobserver variability showed no statistical difference between the mean counts achieved by the observers, and differences in classification of sections as "high" or "low" CD45 counts were seen in $< 10\%$ of cases. No statistical difference was observed in the average CD45 counts obtained from bronchial biopsy specimens compared with those obtained by other routes, indicating that the observed differences were not the result of sampling from

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Table 1—*Summary of Demographic, Clinical, and Survival Details of Study Cases*

Parameter	Study Cases
Sex	
Male	38
Female	26
Age, median (range), y	66 (38-80)
Stage	
Limited	22
Extensive	30
Unknown	12
ECOG performance status	
0	7
1	20
2	13
3	7
4	4
Unknown	13
Chemotherapy	
Yes	41
No	11
Unknown	12
Radiotherapy	
Yes	19
No	33
Unknown	12
Specimen type	
Transthoracic biopsy	14
Bronchial biopsy	43
Resection	4
Mediastinal biopsy	3
Survival, median (range), d	232 (4-3703)

Data are presented as No. unless indicated otherwise. ECOG = Eastern Cooperative Oncology Group.

smoking-related bronchial-associated lymphoid tissue (data not shown).

Kaplan-Meier survival plots were devised by dividing the study group around the average value, which was rounded up to 40 ($CD45_{40}$: CD45 ≤ 40, n = 34; CD45 > 40, n = 30) (Fig 3). Only 3% of patients with a CD45 < 40 survived to 1,000 days compared with 27% of those with a CD45 > 40 ($P < .009$). No relationship between $CD45_{40}$ and age (analysis of variance $P = .63$), sex ($P = .36$), stage ($P = .17$), or treatment by chemotherapy ($P = .12$) or radiotherapy ($P = .28$) was identified (χ^2 test). There was a suggestion of a possible association between the CD45 count and PS, with a bias toward patients with CD45 ≤ 40 tending to have a PS > 2 ($P = .04$, χ^2 test for trend $P = .006$). This finding was examined further by Cox regression analysis. Although PS was found to be a significant predictor of overall survival ($P = .014$), there is little to indicate that PS and CD45 are highly correlated, because both were found to be significant in the model with age (PS: $P = .014$; CD45: $P < .001$; age: $P = .049$).

CD45 Counts and Survival in Patients With Better PS

PS is often used to stratify patients likely to benefit from radical therapy as opposed to palliative or best supportive care. We repeated the analysis by examining the relationship between survival and the CD45 count in patients with a recorded PS of 0 to 2 to determine if the CD45 count was of prognostic value in patients who would be considered for radical therapy. Kaplan-Meier plots showed that for the 40 patients with good PS (≤2), a CD45 score < 40 was associated with a significantly shorter survival (Fig 4) compared with those with higher scores ($P < .0001$).

Discussion

SCLC is a highly aggressive tumor with a poor prognosis that has frequently widely disseminated by the time of diagnosis. For many years, this type of lung cancer has been recognized as systemically influencing

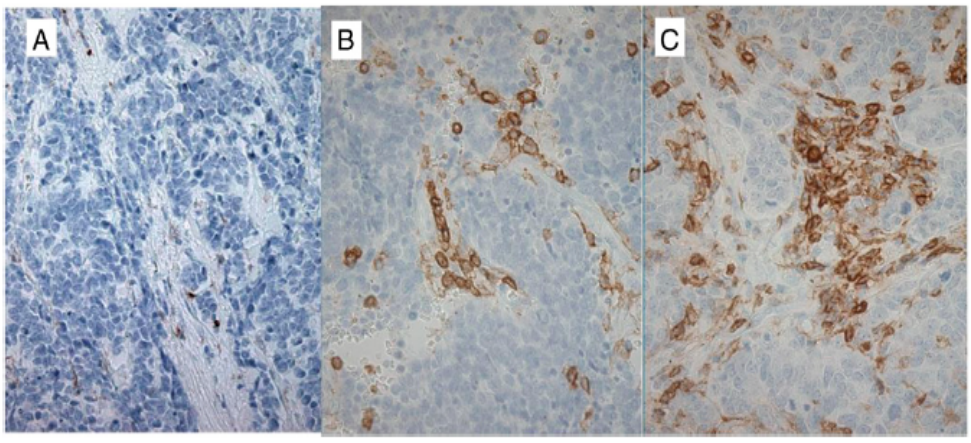

Figure 1. A-C, Photomicrographs from three cases of small cell lung cancer from the study (original magnifications × 400). The pictures illustrate sections of small cell carcinoma stained immunohistochemically to identify the presence of $CD45^+$ cells. The cases shown demonstrate a range of $CD45^+$ (brown staining) cells associated with the tumors, with none being identified in the field shown in A.

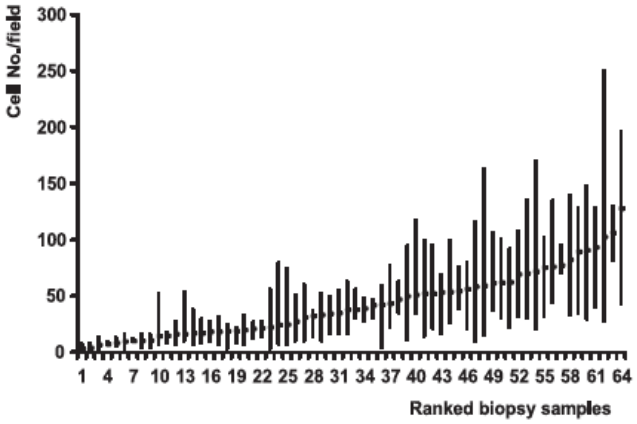

FIGURE 2. Graphic illustration of the mean CD45 count for each case, with the bars indicating the minimal to maximal range of values obtained for the fields counted. The cases are ranked from those with the lowest mean score to the highest.

the function of the immune system. Although patients may respond to chemotherapy and/or radiotherapy, overall survival remains poor,[4] and subsequent relapse and death are the usual outcome. Predictors of poor outcome and survival have centered on traditional factors, such as the stage of the tumor and the patient's PS.[6]

Histologic assessments of local immune/inflammatory responses to tumors as a marker of prognosis have been studied in different tumors, with various results. Previous studies have identified an association between improved prognosis and inflammatory cell infiltrates in melanoma,[19] seminoma,[20] and colorectal carcinoma,[21] and infiltrates were found to be less marked in thyroid tumors with poorer prognosis.[22] In the lung, previous studies have suggested a link between inflammatory cell infiltrates and prognosis in non-small cell carcinoma,[23,24] but, to our knowledge, the relationship between immune cell infiltrates and prognosis has not been examined previously in SCLC.

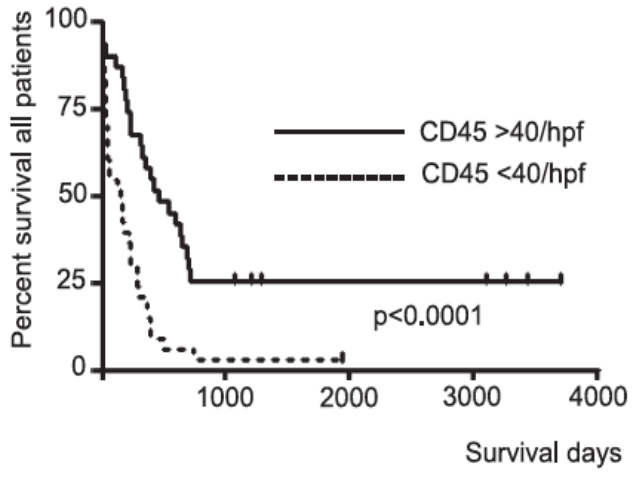

FIGURE 3. Kaplan-Meier survival plot derived for all 64 patients included in the study comparing those with a $CD45_{40}$ count greater than or less than the median value of 40 cells per × 400 magnification field. hpf = high-power field.

Earlier pilot studies conducted in our laboratory have indicated that the majority of inflammatory cells associated with small cell carcinomas are CD3-positive T cells or CD68-positive macrophages, with only a few cases showing small numbers of CD20-positive B cells. With this project, we aimed to determine if a simple assessment of inflammatory cell numbers in association with the tumor might have any relationship to survival. To this end, we elected to use CD45 as our marker because this would function as a paninflammatory cell marker and because it is a reagent widely used in routine diagnostic pathology laboratories.

The data we present are from a relatively small retrospective series of patients with SCLC and we accept that some caution in the interpretation is necessary given the relatively small number of patients with high PS (PS 3: n = 7; PS 4: n = 4). The data, however, suggest that the number of CD45 cells associated with the tumor has a predictive effect on overall survival that is independent of stage and performance score for the patient. Intriguingly, the data also suggest that for patients with good PS (≤ 2), $CD45_{40}$ appears to be a highly significant predictor of survival, with the score obtained being able to potentially predict those with poorer outcomes. The survival curves do appear to show an early, steep fall in survival in the $CD45_{40} < 40$ group that is independent of stage and PS. The reasons for this are not clear and our current study is too small to permit subgroup analysis, but this does support the hypothesis that this observation has clinical relevance in patients with small cell carcinoma.

Previous studies in other tumor types have specifically identified infiltrating inflammatory cells as "intratumoral" or "peritumoral" in location.[22] We found such an approach impossible to adopt in the small diagnostic biopsy specimens available and took the view that inflammatory cells within small biopsy fragments of tissue could reasonably be regarded as "tumor associated" because their recruitment and retention to the tissue at that site was likely to be related to the presence of the tumor. One of the attractions of the method we used is that it uses a scoring system based on standard clinical pathology laboratory immunohistochemical staining with antisera to CD45. This immunohistochemical stain is often carried out as part of the routine diagnostic work-up of these biopsy specimens when the possibility of small cell carcinoma is being considered. The assessment of cell number is relatively straightforward, being based on counting positive cells in hpfs from fragments of tissue containing tumor. This raises the exciting prospect that prognostic information can be obtained easily by the reporting pathologist without the requirement for additional complex investigations.

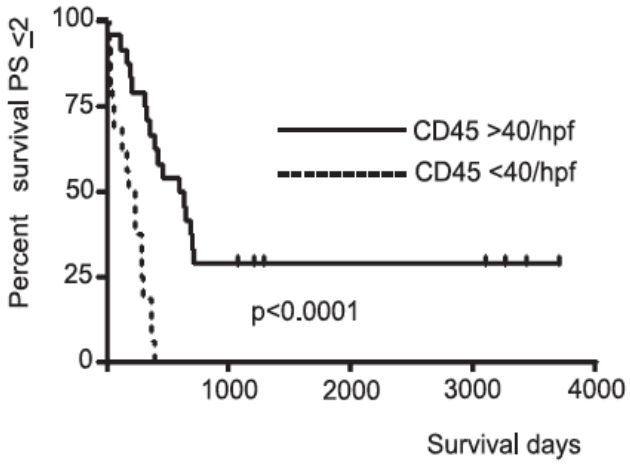

FIGURE 4. Kaplan-Meier survival plot derived for patients with PS ≤2 comparing those with a $CD45_{40}$ count greater than or less than the median value of 40 cells per ×400 magnification field. PS = performance status. See Figure 3 legend for expansion of other abbreviations.

CONCLUSIONS

The number of patients studied is relatively small and the historical data we had available did not include TNM staging, which has been found to provide better prognostic information than previous staging systems for SCLC.[25] The data do strongly suggest that this is an area requiring further investigation. Many patients with SCLC clearly have mediastinal and hilar nodal enlargement at presentation. They are now frequently investigated using mediastinal sampling techniques, such as endobronchial ultrasound-guided fine-needle aspiration to sample the enlarged nodes.[26,27] This development, in our experience, has significantly reduced the number of patients for whom the diagnosis is obtained histologically from the primary tumor mass. Cytologic aspirates from nodes do not provide the types of specimens that allow the assessment we describe. Given our preliminary data, a prospective study is required, using patients who do undergo biopsy for histologic assessment, to determine whether the additional prognostic information that can be obtained from CD45 count necessitates a return to more traditional investigative approaches.

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Dr Howie: contributed to the supervision of laboratory work, data analysis, editing, and approval of the manuscript.
Dr Wallace: contributed to the study design, checking of all pathology, data analysis, writing, and approval of the manuscript.
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Other contributions: This work was performed in the University of Edinburgh, the Royal Infirmary of Edinburgh, and King's College London.

REFERENCES

1. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol*. 2007;18(3):581-592.
2. Franceschi S, Bidoli E. The epidemiology of lung cancer. *Ann Oncol*. 1999;10(suppl 5):S3-S6.
3. Ettinger DS. Overview and state of the art in the management of lung cancer. *Oncology (Williston Park)*. 2004;18(7)(suppl 4):3-9.
4. El Maalouf G, Rodier JM, Faivre S, Raymond E. Could we expect to improve survival in small cell lung cancer? *Lung Cancer*. 2007;57(suppl 2):S30-S34.
5. Govindan R, Page N, Morgensztern D, et al. Changing epidemiology of small-cell lung cancer in the United States over the last 30 years: analysis of the surveillance, epidemiologic, and end results database. *J Clin Oncol*. 2006;24(28):4539-4544.
6. Bremnes RM, Sundstrom S, Aasebø U, Kaasa S, Hatlevoll R, Aamdal S; Norweigian Lung Cancer Study Group. The value of prognostic factors in small cell lung cancer: results from a randomised multicenter study with minimum 5 year follow-up. *Lung Cancer*. 2003;39(3):303-313.
7. Ellyard JI, Simson L, Parish CR. Th2-mediated anti-tumour immunity: friend or foe? *Tissue Antigens*. 2007;70(1):1-11.
8. Graus F, Keime-Guibert F, Reñe R, et al. Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients. *Brain*. 2001;124(Pt 6):1138-1148.
9. Manley GT, Smitt PS, Dalmau J, Posner JB. Hu antigens: reactivity with Hu antibodies, tumor expression, and major immunogenic sites. *Ann Neurol*. 1995;38(1):102-110.
10. Graus F, Dalmou J, Reñé R, et al. Anti-Hu antibodies in patients with small-cell lung cancer: association with complete response to therapy and improved survival. *J Clin Oncol*. 1997;15(8):2866-2872.
11. Masuno T, Ikeda T, Yokota S, Komuta K, Ogura T, Kishimoto S. Immunoregulatory T-lymphocyte functions in patients with small cell lung cancer. *Cancer Res*. 1986;46(8):4195-4199.
12. Fischer JR, Schindel M, Stein N, et al. Selective suppression of cytokine secretion in patients with small-cell lung cancer. *Ann Oncol*. 1995;6(9):921-926.
13. Fischer JR, Schindel M, Bülzebruck H, Lahm H, Krammer PH, Drings P. Decrease of interleukin-2 secretion is a new independent prognostic factor associated with poor survival in patients with small-cell lung cancer. *Ann Oncol*. 1997;8(5):457-461.
14. Johnston-Early A, Cohen MH, Fossieck BE Jr, et al. Delayed hypersensitivity skin testing as a prognostic indicator in patients with small cell lung cancer. *Cancer*. 1983;52(8):1395-1400.

15. Taylor RC, Patel A, Panageas KS, Busam KJ, Brady MS. Tumor-infiltrating lymphocytes predict sentinel lymph node positivity in patients with cutaneous melanoma. *J Clin Oncol*. 2007;25(7):869-875.
16. Iwamoto M, Shinohara H, Miyamoto A, et al. Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. *Int J Cancer*. 2003;104(1):92-97.
17. Sato E, Olson SH, Ahn J, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A*. 2005;102(51):18538-18543.
18. Yu P, Fu YX. Tumor-infiltrating T lymphocytes: friends or foes? *Lab Invest*. 2006;86(3):231-245.
19. Bröcker EB, Kolde G, Steinhausen D, Peters A, Macher E. The pattern of the mononuclear infiltrate as a prognostic parameter in flat superficial spreading melanomas. *J Cancer Res Clin Oncol*. 1984;107(1):48-52.
20. Mostofi FK, Sesterhenn I. Plenary lecture: lymphocytic infiltration in relationship to urologic tumors. *Natl Cancer Inst Monogr*. 1978;49(49):133-141.
21. Murphy J, O'Sullivan GC, Lee G, et al. The inflammatory response within Dukes' B colorectal cancers: implications for progression of micrometastases and patient survival. *Am J Gastroenterol*. 2000;95(12):3607-3614.
22. Ugolini C, Basolo F, Proietti A, et al. Lymphocyte and immature dendritic cell infiltrates in differentiated, poorly differentiated, and undifferentiated thyroid carcinoma. *Thyroid*. 2007;17(5):389-393.
23. Johnson SK, Kerr KM, Chapman AD, et al. Immune cell infiltrates and prognosis in primary carcinoma of the lung. *Lung Cancer*. 2000;27(1):27-35.
24. Bremnes RM, Al-Shibli K, Donnem T, et al. The role of tumor-infiltrating immune cells and chronic inflammation at the tumor site on cancer development, progression, and prognosis: emphasis on non-small cell lung cancer. *J Thorac Oncol*. 2011;6(4):824-833.
25. Ignatius Ou SH, Zell JA. The applicability of the proposed IASLC staging revisions to small cell lung cancer (SCLC) with comparison to the current UICC 6th TNM edition. *J Thorac Oncol*. 2009;4(3):300-310.
26. Herth FJ, Eberhardt R, Vilmann P, Krasnik M, Ernst A. Real-time endobronchial ultrasound guided transbronchial needle aspiration for sampling mediastinal lymph nodes. *Thorax*. 2006;61(9):795-798.
27. Wallace WAH, Monaghan HM, Salter DM, Gibbons MA, Skwarski KM. Endobronchial ultrasound-guided fine-needle aspiration and liquid-based thin-layer cytology. *J Clin Pathol*. 2007;60(4):388-391.

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Small cell lung cancer tumour cells induce regulatory T lymphocytes, and patient survival correlates negatively with FOXP3$^+$ cells in tumour infiltrate

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Small cell lung cancer (SCLC) kills at least one person every 2 hr in the United Kingdom. Some patients do relatively well but most have rapidly progressive disease. There is no effective treatment and overall 2-year survival is less than 5%. Patients with SCLC have poorly understood local and systemic immune defects and can be immunocompromised. As CD4$^+$ T lymphocytes coordinate and regulate immunity, a better understanding of interactions between SCLC tumour cells and CD4$^+$ T cells may lead to effective molecular immunotherapy. We show that some, but not all, SCLC tumour cell lines secrete molecules that induce IL-10 secretion by and *de novo* differentiation of functional CD4$^+$CD25$^+$FOXP3$^+$CD127^{lo}Helios$^-$ regulatory T (Treg) cells in healthy blood lymphocytes. FOXP3$^+$ T cells were found in SCLC tumour biopsies, and patients with higher ratios of FOXP3$^+$ cells in tumour infiltrates have a worse survival rate. The inhibitory effect of SCLC tumour cells was not affected by blocking IL-10 receptor or TGF-β signalling but was partially reversed by blocking IL-15, which is reported to be involved in human Treg cells induction. IL-15 was secreted by SCLC cells that inhibited CD4$^+$ T-cell proliferation and was present in SCLC biopsy tumour cells. These novel findings demonstrate that SCLC tumour cells can induce CD4$^+$ T-cell-mediated immunosuppression. This gives a potential mechanism by which SCLC tumour cells may downregulate local and systemic immune responses and contribute to poor patient survival. Our data suggest that IL-15 and Treg cells are potential new therapeutic targets to improve immune response and patient survival in SCLC.

Small cell lung cancer (SCLC), which constitutes 10–20% of all lung cancers, is particularly aggressive with wide-spread metastasis at presentation. At least one person dies of SCLC every 2 hr in the United Kingdom, and, despite treatment, 2-year survival is less than 5%.[1,2] Novel therapeutic strategies are urgently required. A minority of patients with SCLC mount immune responses to tumour-associated antigens and have a more favourable prognosis.[3] Conversely, most patients with SCLC are immunocompromised with poorly understood local and systemic immune defects that correlate with worse morbidity and mortality.[4,5]

The immune system protects the host from tumour development, growth and metastasis,[6–8] and immune suppression[9] (including that caused by HIV infection[10]) promotes lung cancer development. CD4$^+$ T lymphocytes play a central role in anti-tumour immune responses.[11] The CD4$^+$ T-cell subset contains both 'helper' T cells, which coordinate acquired immune effector responses and generate cell-mediated and humoral effector immunity, and 'regulatory' T (Treg) cells, which downregulate immune effector responses.[12] Treg cells can inhibit activation, expansion and effector function of other T cells.[13] Two broad Treg subsets that express the transcription factor forkhead box protein P3 (FOXP3) are described *in vivo*: 'natural' Treg (nTreg) cells, which are released from the thymus, and 'induced' Treg (iTreg) cells, which are differentiated from resting CD4$^+$ T cells as part of the response to antigen challenge. CD127, the α-chain of the IL-7 receptor, has been shown to be a marker to discriminate between human CD4$^+$ regulatory and activated T cells, and its expression inversely correlates with FOXP3 and suppressive function.[14,15]

Helios protein, a member of the Ikaros transcription factor family, has been reported to distinguish Treg subsets with nTreg cells being positive and iTreg cells being negative for Helios protein.[16] However, Helios expression has been recently observed in peripherally induced FOXP3$^+$ regulatory cells[17] and is associated with T-cell activation and proliferation.[18]

The role of Treg cells in cancer is not fully understood and differs with the type of tumour. FOXP3$^+$ T cells were

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found to infiltrate human non-SCLC and ovarian cancer[19] and have been subsequently identified in a number of cancers. However, the effect of $FOXP3^+$ T-lymphocyte infiltration on patient survival is controversial and has been variously reported to have no prognostic effect,[20,21] to be associated with improved survival[13,22,23] or to predict poor survival.[24,25] Understanding how Treg cells contribute to cancer progression may need to be determined on a tumour-type basis.

Progress in understanding SCLC pathobiology has been hampered by lack of animal models. To determine how SCLC tumour cells can affect immune system responses, we investigated the effects of tumour cell lines on $CD4^+$ T cells isolated from healthy human blood and studied SCLC biopsies and matching clinical data from a cohort of 65 patients. Here, we show for the first time that some, but not all, SCLC tumour cell lines constitutively secrete molecules that suppress proliferation of activated $CD4^+$ T cells from healthy donors *in vitro* by inducing *de novo* $CD4^+CD25^+FOXP3^+CD127^{lo}Helios^-$ Treg cell differentiation. The suppressive effect is independent of IL-10R or TGF-β signalling but can be partially reversed by IL-15 blockade. IL-15 is secreted by those SCLC tumour cells that induce Treg cells. IL-15 is present in tumour cells in SCLC biopsies, and patients with higher ratios of $FOXP3^+$ cells in the immune infiltrate of their tumours have a significantly worse survival rate of 1,000 days post-diagnosis. These data indicate that IL-15 and Treg cell function may be new targets for therapy to improve immunosuppression and survival in SCLC.

Material and Methods

Ethical approval

The use of healthy blood donors and of patient material and the clinical data were approved by the Lothian Research Ethics committee.

SCLC cells

Mycoplasma-free cell banks of SCLC cell lines, NCI-H69, NCI-H345 (ECACC, Health Protection Agency, Porton Down, UK) and NCI-H510 (ATCC, LGC Standards, Teddington, UK) were grown and stored in liquid nitrogen. Cells in suspension at 2–5 × 10^5 ml^{-1} were passaged up to ten times in 75-cm^2 flasks (Sigma, Poole, UK) in complete RPMI (RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum, 5 μg/ml L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin; all obtained from Invitrogen, Paisley, UK) at 37°C in a humidified 5% CO_2 incubator.

H69 SCLC cell conditioned medium and cell lysate

H69 cells were washed and resuspended at 2.5 × 10^6 ml^{-1} in 10-ml serum-free Iscove's modified Dulbecco's medium (IMDM; Sigma) supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin in 25-cm^2 tissue culture flasks (Sigma) for 72 hr. Conditioned medium (CM) was centrifuged at 1,000*g* for 5 min to remove cells. To lyse cells, the pellet was resuspended in lysis buffer [10 ml phosphate buffered saline (PBS), 1 Mimi protease inhibitor cocktail tablet (Roche, Welwyn Garden City, UK), 1 mM Na_3VO_4 and 100 μl octylphenoxypolyethoxyethanol (IGEPAL, Sigma)] for 30 min on ice.

Isolation of peripheral blood mononuclear cells

Human peripheral blood from normal healthy donors was layered onto Lymphoprep™ (Axis-Shield, Cambridgeshire, UK) and centrifuged at 2,000*g* for 20 min. Cells from the interface were washed thrice with PBS by centrifugation at 1,000*g* to remove platelets.

Mixed lymphocyte reactions

Two-way mixed lymphocyte reactions (MLRs) were established from peripheral blood mononuclear cells (PBMCs) of unrelated donors. H69 SCLC cells were washed, resuspended in PBS (10^6 cells per milliliter), incubated with mitomycin-C (50 μg/ml; Sigma) for 3 hr at 37°C, washed thrice in PBS and resuspended in complete IMDM (10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin). In a 96-well plate (Sigma), 5 × 10^4 PBMCs per donor per well were combined with mitomycin-C-treated SCLC cells at a 1:1 ratio in 200 μl complete IMDM at 37°C in 5% CO_2 in a humidified incubator for 72 hr.

Naïve/$CD4^+$ T-cell activation

$CD4^+$ T cells were isolated from PBMCs by negative selection on MACS™ columns using the $CD4^+$ T-cell isolation kit (Miltenyi Biotec, Bisley, UK). Purity was 95.6% ± 0.9%, which was evaluated by flow cytometry. Naïve $CD4^+$ T cells were purified from PBMCs using naïve T-cell isolation kit II (Miltenyi Biotec). The purity of $CD4^+CD45RA^+$ cells was above 95%. Purified naïve/$CD4^+$ T cells, at 2 × 10^5 cells per well, were stimulated with 1.25 μg per well immobilised anti-CD3 (OKT3, pre-coated for 6 hr at 37°C) and 1 μg/ml soluble anti-CD28 (CD28.2) monoclonal antibodies (e-Bioscience, Hatfield, UK) or with Dynabeads CD3/CD28 T-cell expander (one bead per cell, Invitrogen) in 1 ml per well complete IMDM in 24-well plates (Millipore, Watford, UK) at 37°C in 5% CO_2 in a humidified incubator for 72 hr.

Cell proliferation

Carboxyfluorescein diacetate succinimidyl ester incorporation. Cells were incubated with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen; 1 μM; Molecular Probes Invitrogen) according to manufacturer's instructions. After 72 hr, cells were harvested, and cell division was estimated by flow cytometry. Data were acquired on a FACSCalibur (BD Biosciences) flow cytometer and analysed with FlowJo™ software.

^{3}H-Thymidine incorporation. Cells were cultured for 48 hr and then pulsed for 24 hr with 1 μCi ^{3}H-thymidine (Perkin Elmer, Cambridge, UK) per well. Cells were harvested with a

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Tomtec (Leamington Spa, UK) cell harvester and ^{3}H-thymidine incorporation counted in a β-plate reader (Wallac, UK).

Cytokine ELISAs

IL-10, IFN-γ, IL-4 and IL-17 were measured by Duo-set ELISAs (R&D systems, Abingdon, UK) and IL-15 by Ready-SET-Go!® (e-Bioscience) according to manufacturers' instructions. Plates were read at 450 nm (650 nm reference) on a Biotek Synergy HT plate reader (Fisher Scientific, Loughborough, UK).

Coculture of SCLC cells and CD4$^+$ T cells

SCLC cells were treated with mitomycin-C as above. In 96-well plates, CD4$^+$ T cells (2×10^5 cells per well) were activated with immobilised anti-CD3 (0.5 μg per well) and soluble anti-CD28 (0.1 μg per well) and cultured alone or in combination with mitomycin-C-treated SCLC cells at a 1:1 ratio.

Transwell cocultures

Polycarbonate 24-well transwell inserts (0.4 μM) and receiver trays (Millipore) were used to physically separate naïve/CD4$^+$ T cells and SCLC cells. Naïve/CD4$^+$ T cells (2×10^5) in the lower chambers were stimulated with immobilised anti-CD3 and soluble anti-CD28 monoclonal antibodies in 0.8 ml complete IMDM. Complete IMDM (0.2 ml) only or containing SCLC cells at T cell/SCLC cell ratios of 1:1, 1:2 or 1:4 was added to the upper chambers.

Treg functional assay

CD4$^+$ T cells isolated from single donors were divided into two aliquots. One aliquot was stimulated as above with anti-CD3/CD28 antibodies and cultured alone or cocultured with SCLC cells at 1:4 ratio in transwells for 3 days. The second aliquot was immediately frozen and stored at −80°C for 3 days in freezing medium (40% complete IMDM with 50% FBS and 10% DMSO; Sigma).

On Day 3, frozen naïve/CD4$^+$ T cells were quickly thawed (37°C, 5 min) and washed by centrifugation in warm complete 3× IMDM. Viable cells (>95%) were counted by trypan blue exclusion and labelled with CFSE. The previously cultured autologous CD4$^+$ T cells were harvested and counted. CFSE-labelled defrosted CD4$^+$ T cells (10^5 cells per well) and the unlabelled cultured autologous CD4$^+$ T cells (2×10^5 cells per well) were cocultured in 24-well plates in the presence of Dynabeads™ CD3/CD28 T-cell expander (Invitrogen). After 72-hr proliferation, cell division of CFSE-labelled CD4$^+$ T cells was analysed by flow cytometry.

Cytokines and cytokine-signalling blockers

IL-15 (20 ng/ml; R&D) was added as indicated. TGF-β signalling was blocked with monoclonal neutralising anti-TGF-β antibody (active against all isoforms) and compared to isotype control antibody at 10 μg/ml (R&D Systems, Abingdon, UK) or with 1 μM of the TGF-β and activin-signalling inhibitor SB 431542 hydrate (Sigma) and vehicle control. IL-10 signalling was blocked using monoclonal blocking anti-human IL-10 Rα antibody compared to isotype control antibody (20 μg/ml; R&D). IL-15 was blocked using monoclonal neutralising anti-IL-15 compared to isotype control antibody (e-Bioscience).

Flow cytometric analysis of protein expression

Surface marker staining used standard protocols with mouse monoclonal antibodies: FITC-anti-CD8, RPE-anti-CD4; RPE-Cy5-anti-CD3 and relevant isotype controls (all obtained from DAKO, UK); PE-anti-CD45RA (HI100) and PE-anti-CD127 (eBioRDR5; both obtained from e-Bioscience). For Treg cell (CD4$^+$CD25$^+$FOXP3$^+$) staining, the human Treg cell detection kit (Miltenyi Biotec) was used. Briefly, CD4$^+$ T cells were stained with FITC-anti-CD4 (VIT4) and PE-anti-CD25 (4E3), fixed and permeabilised, blocked with FcR blocking reagent and stained with APC-anti-FOXP3 (3G3) or isotype control. Treg cell was also stained with FITC-anti-CD4 (VIT4), PE-anti-CD127 (eBioRDR5) and APC-anti-FOXP3 (3G3). In some experiments, freshly isolated CD4$^+$ T cells were labelled with CFSE as above, cocultured in transwells with H69 cells 1:4 for 3 days, then fixed and permeabilised, blocked with FcR blocking reagent, stained with PE-anti-mouse/human Helios protein (22F6; Biolegend, UK) and APC-anti-human FOXP3 (3G3) or isotype control and analysed by Flow Cytometry. Data were acquired on a FACSCalibur (BD Biosciences) flow cytometer and analysed with FlowJo™ software.

Immunofluorescence and immunohistochemistry

Paraffin-embedded formalin-fixed lung tumour biopsies from patients with SCLC were obtained from the Department of Pathology, Royal Infirmary of Edinburgh. Patient details and survival data were collected by P.H. Sixty-five patients (26F, 39M; median age 66 years) were studied; median survival time was 235 days.

Sections (3 μm) were antigen retrieved in BORG Decloaker pH9.5 (1,000 W microwave, 10 min maximum power; Biocare Medical, Walnut Creek, CA). For immunofluorescence, the sections were blocked (5% goat serum, 30 min), incubated with rabbit-anti-CD3 (DAKO) and monoclonal mouse-anti-FOXP3 (e-Bioscience) diluted in 5% goat serum for 1 hr, washed and incubated with Alexa-488-goat anti-rabbit IgG and Alexa-568-goat anti-mouse IgG (both 1:1000, Invitrogen) for 30 min followed by 4,6-diamidino-2-phenylindole (DAPI, 0.1 μg/ml) to stain nuclei. The sections were mounted in aqueous mounting medium (DAKO) and analysed on a Leica TCS SP5 confocal microscope.

For immunohistochemistry, the sections (3 μm) were antigen retrieved as above, blocked with 3% hydrogen peroxide for 15 min (Sigma), washed with TBS, loaded into a Shandon Sequenza® slide rack (Fisher Scientific, Loughborough UK) and 100 μl primary antibody (mouse monoclonal anti-CD3, anti-CD4, anti-CD45; Novocastra Reagents, Leica Microsystems, Wetzlar, Germany) or anti-IL15 (Abcam, Cambridge,

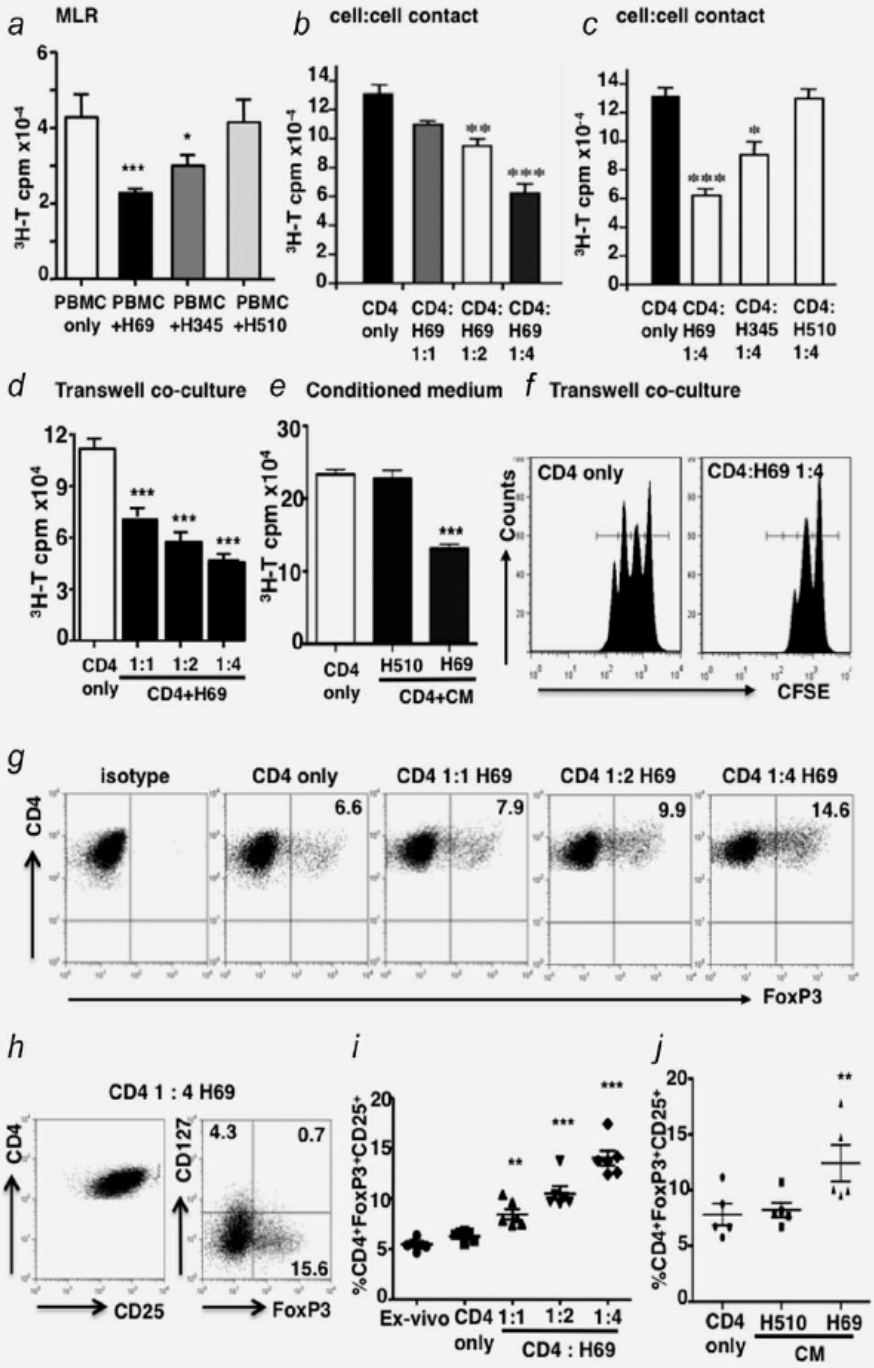

Figure 1. H69 SCLC cells and their soluble molecules inhibit healthy blood-derived $CD4^+$ T-cell proliferation and induce $CD4^+CD25^+FOXP3^+CD127^{lo}$ Treg cells. (*a*) MLR inhibition by mitomycin-C-treated H69 and H345 cells. Mean ± SEM of seven experiments. $*p < 0.05$, $***p < 0.0001$. (*b*) $CD4^+$ T-cells activated by CD3/28 ligation in the presence of different ratios of mitomycin-C-treated H69 cells. (*c*) $CD4^+$ T cells activated by CD3/28 ligation in the presence of H69, H510 and H345 cells at 1:4 ratio. (*d*) H69 cells separated from $CD4^+$ T cells in transwell cocultures inhibit anti-CD3/CD28-induced ^{3}H-thymidine incorporation; mean ± SEM of eight donors; $***p < 0.0001$. (*e*) H69 CM reduces $CD4^+$ T-cell proliferation induced by CD3/CD28 ligation; mean ± SEM of seven donors; $***p < 0.0001$. (*f*) Representative flow cytometry showing that transwell coculture at 1:4 $CD4^+$ T cells:SCLC cells reduces percent of cells undergoing division and number of division cycles completed by CFSE-labelled $CD4^+$ T cells. (*g*) Representative flow cytometry showing increased Treg cells in purified $CD4^+$ T cells activated in transwell cultures for 72 hr by CD3/CD28 ligation alone or with different ratios of H69 SCLC cells; cells were stained with anti-CD4, anti-CD25 and anti-FOXP3 antibodies and gated on CD25 expression. (*h*) $CD4^+$ T cells cocultured with H69 (1:4 ratio of $CD4^+$:H69) stained with anti-CD4, anti-CD25, anti-CD127 and anti-FOXP3. (*i*) Data from six donors cultured as in (*a*) showing increased percent of $CD25^+FOXP3^+$ cells in the $CD4^+$ gate with increasing numbers of H69 cells. $**p < 0.01$, $***p < 0.0001$. (*j*) Data from four donors showing that CD3/CD28 ligation in 40% H69 CM increases Treg cells differentiation; $**p < 0.01$.

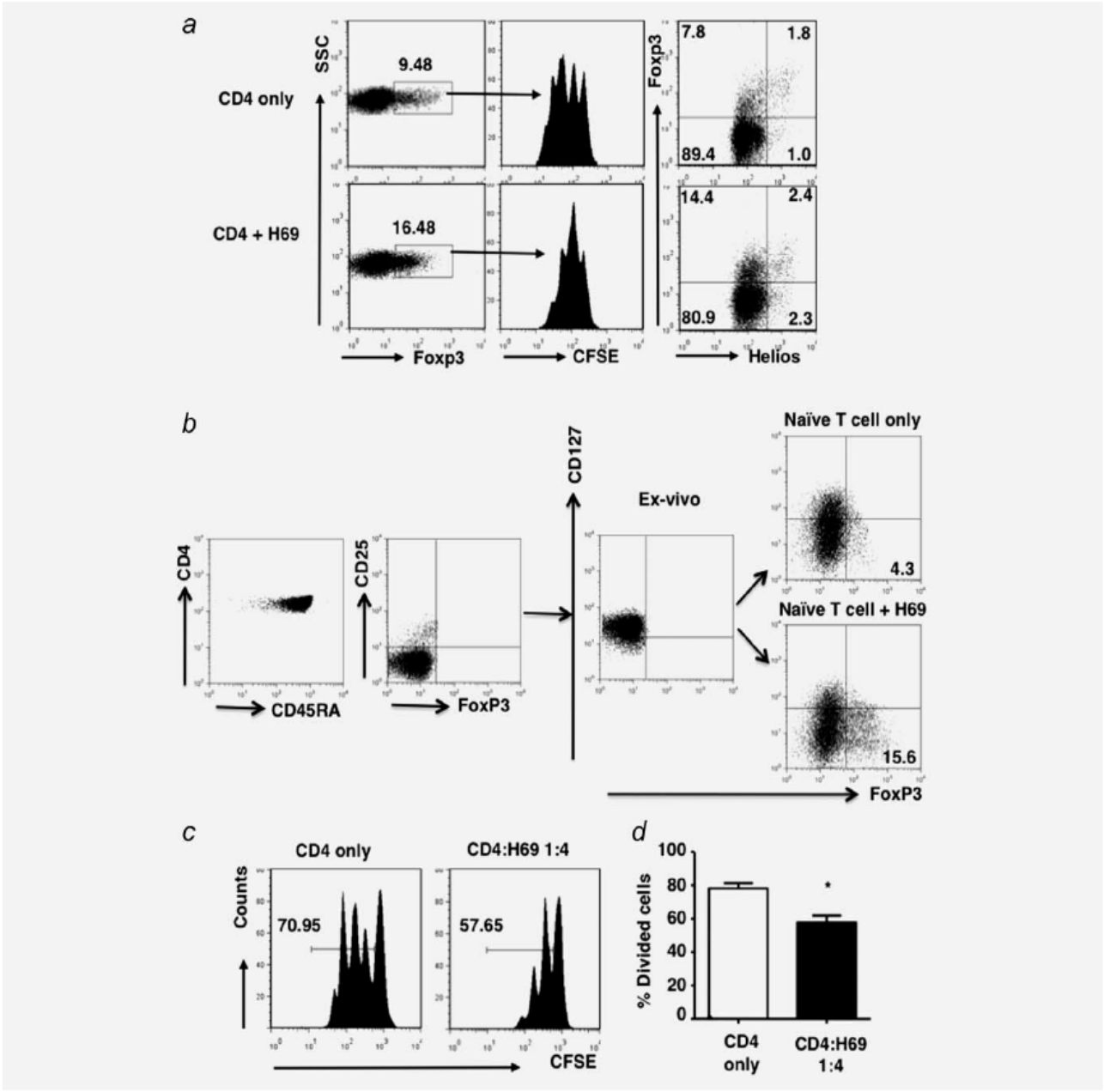

Figure 2. The induced Treg cells were derived from differentiation of naïve T cells and were functional suppressive. (*a*) Representative flow cytometry of purified $CD4^+$ T cells in transwell cultures activated as in Fig. 1*g* with 1:4 CD4:H69 cells showing increased $FOXP3^+$ cells despite reduced cell proliferation. The increased $FOXP3^+$ cells are $Helios^-$. (*b*) Purified naïve T cells and the cultured cells as in (*a*) stained with anti-CD4, anti-CD45RA, anti-CD25, anti-CD127 and anti-FOXP3. Representative flow cytometry showing H69 cell-induced $FOXP3^+CD127^{lo}$ Treg cells population from naïve T cells. (*c*) Representative flow cytometry showing that unlabelled cells previously activated in transwell cultures for 72 hr by CD3/CD28 ligation with H69 SCLC cells at 1:4 T:SCLC ratio inhibit CD3/CD28 ligation-induced proliferation of autologous CFSE-labelled $CD4^+$ T cells. (*d*) Percent of activated $CD4^+$ T cells undergoing division is reduced when cocultured with autologous unlabelled cells previously activated with H69 SCLC cells in transwells as in (*e*); mean ± SEM of three donors. $*p < 0.05$.

UK) added to each slide overnight at 4°C. Slides were rinsed twice with TBS and EnVision™ developing reagents (DAKO, Ely, UK) used as per manufacturer's instructions. Slides were washed with TBS, and 100 µl 3,3′-Diaminobenzidine (DAB, DAKO) solution was applied for 5 min. Cytospins of H69 and H510 cells were air dried and fixed in 90% anhydrous acetone/10% methanol. IL-15 immunohistochemistry was performed on cytospins as above but without antigen retrieval.

Statistics

Data are expressed as mean ± SEM. The statistical significance of the difference between two groups was performed using Student's *t*-test. For multiple group comparisons, one-way ANOVA with Tukey's post-test was performed. Correlation between values was evaluated using non-parametric Spearman's rank correlation. Kaplan–Meyer survival curves were plotted, and all statistical analysis was performed using

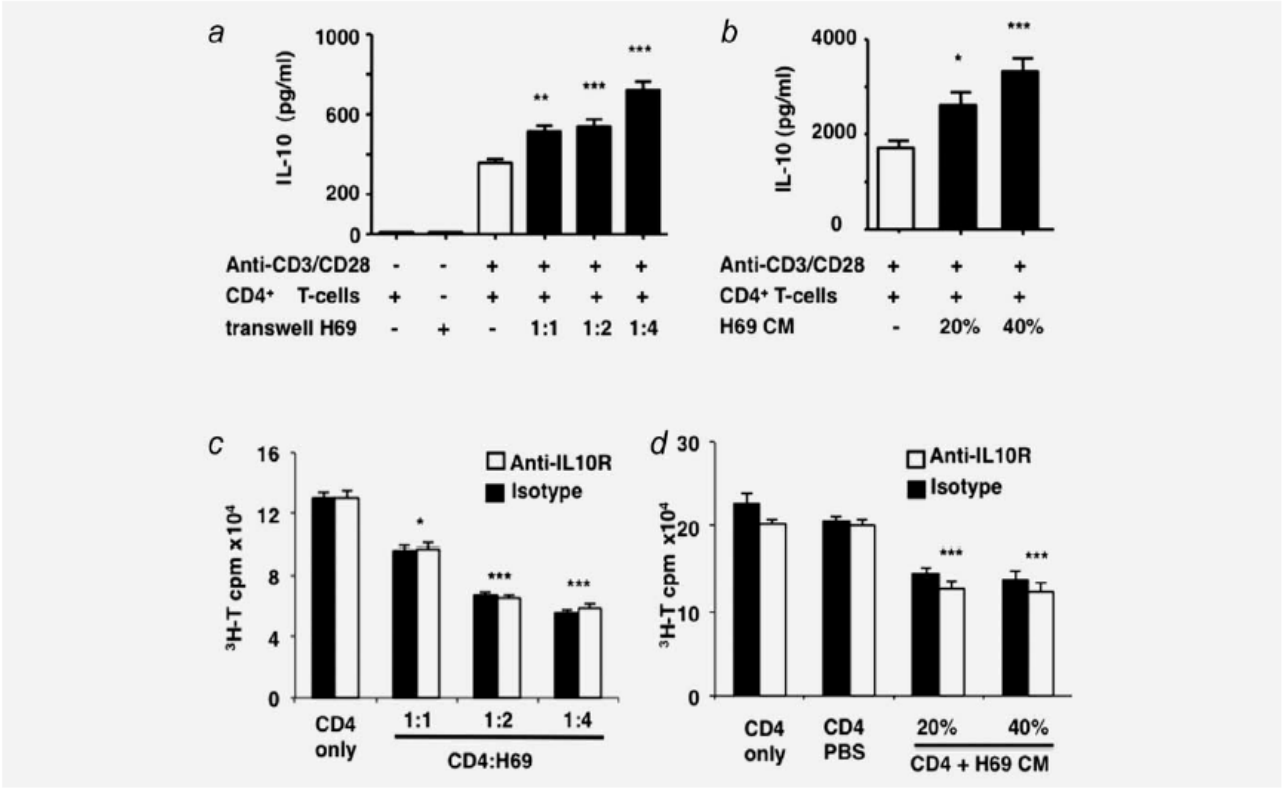

Figure 3. SCLC-induced inhibition of proliferation is IL-10 independent although secretion of IL-10 is increased. (*a* and *b*) $CD4^+$ T-cell IL-10 production after 72 hr CD3/CD28 ligation in (*a*) transwell cocultures or (*b*) H69 CM; mean ± SEM of six donors; $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$. (*c* and *d*) Proliferation of $CD4^+$ T cells after 72 hr CD3/CD28 ligation in (*c*) transwell cocultures or (*d*) H69 CM in the presence of anti-IL-10 neutralising antibody or isotype control; mean ± SEM of three donors; $*p < 0.05$, $***p < 0.0001$.

GraphPad Prism™ 5 (Graph Software, San Diego, CA). Probability values (p) <0.05 were considered statistically significant.

Microarray analysis

All comparative analysis was carried out by the Bioinformatics Team in the BHF Centre for Research Excellence, University of Edinburgh. Expression data were downloaded from the Gene Expression Omnibus. All result sets were derived from variants of the Affymetrix Human Genome U133 GeneChip. Expression values were converted to the linear scale where appropriate. Annotation for the probe identifiers on these chips was derived from appropriate annotation packages provided by the Bioconductor software suite (www.bioconductor.org). All data were output to a relational database and examined by use of a web-based query builder. Although the vast majority of probe identifiers were comparable between chips, this web interface allowed qualitative comparison of expression for different probe IDs mapping to the same gene *via* their gene IDs from Entrez (cite entrez gene PMID 15608257). Because these data were derived from disparate experiments and not cross-normalised, this methodology allows only for a coarse comparison of expression levels.

Results

SCLC-secreted soluble molecules inhibit $CD4^+$ T-cell proliferation through preventing activated $CD4^+$ T cells from entering cell cycle

Three SCLC tumour cell lines were tested to evaluate the effects on lymphocyte proliferation. Coculture of two-way PBMC MLRs (driven by recognition of disparate MHC-II antigens on antigen-presenting cells, which are recognised by $CD4^+$ T lymphocytes) with non-dividing (mitomycin-C treated) H69 and H345 significantly reduced proliferation, whereas H510 did not (Fig. 1*a*).

To investigate the direct effects on $CD4^+$ T lymphocytes, negatively selected, purified $CD4^+$ T-cells from healthy donors were cultured alone or with 1:1 mitomycin-C-treated H69 cells and activated by ligation of CD3 and CD28. Proliferation was significantly suppressed by mitomycin-C-treated H69 cells after 72 hr in a dose-dependent fashion (Fig. 1*b*). To determine whether the different effects between the SCLC cell lines on MLR proliferation were the same in this system of direct $CD4^+$ T cell activation, the cell lines were compared. Mitomycin-C-treated H69 and H345 cells inhibited proliferation, but H510 had no effect (Fig. 1*c*). To determine whether or not the inhibition required cell–cell contact, $CD4^+$ T cells were stimulated for 72 hr by CD3/CD28

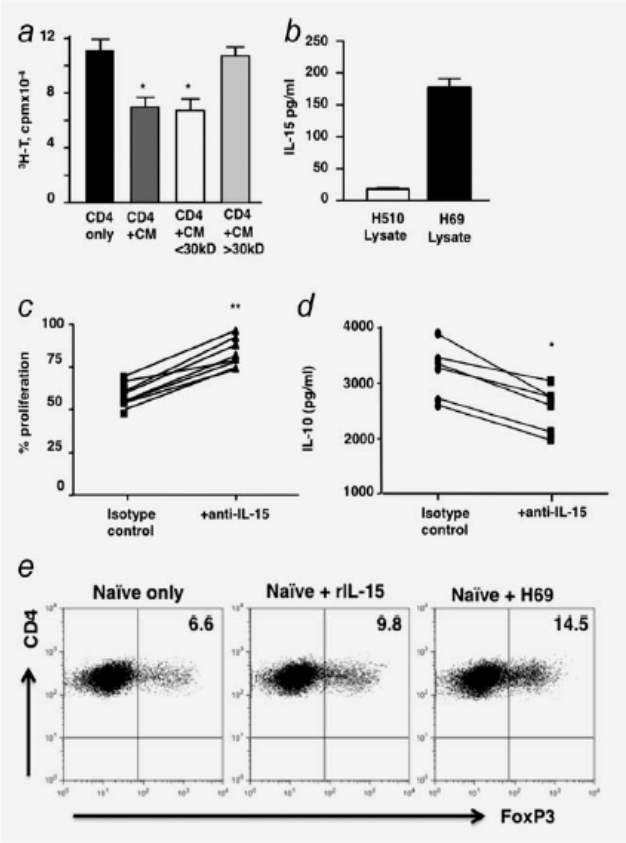

Figure 4. IL-15 is produced by H69 but not H510 SCLC cells and contributes to suppression of CD4$^+$ T-cell proliferation and increased IL-10 secretion induced by H69 CM. (*a*) The soluble activity in H69 CM responsible for inhibition of CD4$^+$ T-cell proliferation is >30 kDa; mean ± SEM of three donors; *$p < 0.05$. (*b*) IL-15 is present in CM and lysate of H69 cells. (*c*) Data from eight donors showing that IL-15-neutralising antibody added to CD4$^+$ T cells activated by CD3/CD28 ligation in 40% CM derived from H69 SCLC cells partially reverses the inhibition of proliferation; **$p < 0.01$. (*d*) Data from six donors showing decreased IL-10 production by CD4$^+$ T cells activated by CD3/CD28 ligation in 40% CM derived from H69 SCLC cells in the presence of IL-15-neutralising antibody or isotype control; *$p = 0.0313$. (*e*) Representative flow cytometry showing Treg cells induced from naïve CD4$^+$ T cells activated by CD3/CD28 ligation for 72 hr alone in the presence of IL-15 or with H69 cells at 1:4 ratio of T:H69 in transwell cultures; cells were stained with anti-CD4, anti-CD25 and anti-FOXP3 antibodies and gated on CD25 expression.

ligation in the bottom of transwells with medium only or with SCLC cells in the upper wells. CD4$^+$ T-cell proliferation was inhibited by H69 cells in a dose-dependent fashion (Fig. 1*d*), indicating that cell–cell contact was not necessary. To assess whether the soluble inhibitory factor was constitutively produced, serum-free CM from H69 or H510 cells was added to cultures of CD4$^+$ T cells stimulated for 72 hr by CD3/CD28 ligation. Proliferation was reduced by the addition of 20% H69-CM but not by 20% H510-CM (Fig. 1*e*).

To determine the nature of the proliferation block, CD4$^+$ T cells were labelled with CFSE immediately after purification and stimulated by CD3/CD28 ligation in transwells with SCLC cells at a ratio of 1:4. When cells divide, exactly half of the fluorescent CFSE is transferred to daughter cells allowing estimation of divisions by flow cytometry. Figure 1*f* shows that both the fraction of dividing CD4$^+$ cells and the number of divisions per cell were significantly reduced.

SCLC-secreted soluble molecules induce *de novo* functional CD4$^+$CD25$^+$FOXP3$^+$CD127^{lo}Helios$^-$ Treg cells

To investigate whether decreased proliferation was due to Treg cell induction, purified CD4$^+$ T cells were activated by CD3/CD28 ligation and cultured alone or with H69 SCLC tumour cells in transwells or in H69 CM for 72 hr, stained and analysed by flow cytometry. The population of Treg cells (CD4$^+$CD25$^+$FOXP3$^+$CD127^{lo}) was increased in transwell cocultures (Figs. 1*g*–1*i*) and by H69 CM (Fig. 1*j*) in a dose-dependent fashion.

To determine whether increased Treg cells was due to expansion of preexisting cells (nTreg cells) or *de novo* differentiation of naïve cells (iTreg cells), first, CFSE-labelled CD4$^+$ T cells were activated as above in transwell cocultures (1:4 of CD4$^+$:H69 cells) for 72 hr. H69 cells increased differentiation of FOXP3$^+$Helios$^-$ cells despite reduced proliferation, and the ratio of FOXP3$^+$Helios$^-$/FOXP3$^+$Helio$^+$ cells increased (Fig. 2*a*). Importantly, Treg cells (CD4$^+$CD25$^+$FOXP3$^+$CD127^{lo}) induction is observed from naïve T cells with a phenotype of CD4$^+$CD45RA$^+$CD25$^-$FOXP3$^-$CD127^{hi} (Fig. 2*b*), suggesting that the Treg phenotype was induced by differentiation of naïve T cells rather than by expansion of preexisting nTreg cells.

To determine whether Treg cells were functional, CD3/CD28-activated CD4$^+$ T cells previously cocultured with SCLC cells (ratio: 1:4) in transwells for 3 days were harvested and cocultured with CFSE-labelled, autologous, unstimulated T cells in the presence of anti-CD3/CD28 beads for 72 hr. Proliferation of CFSE-labelled cells was significantly suppressed by the presence of unlabelled CD4$^+$ T cells previously cocultured with SCLC cells (Figs. 2*c* and 2*d*).

The suppressive effect is not dependent on IL-10 or TGF-β

To examine whether proliferation inhibition was associated with immunosuppressive cytokines, levels of IL-10 and TGF-β were determined in supernatants of CD4$^+$ T cells activated by CD3/CD28 ligation in transwell coculture or in H69 SCLC CM. No TGF-β (activated or latent) was detected above background, and neither antibody-mediated inhibition nor pharmacological inhibition of TGF-β signalling had any effect on the inhibition of proliferation (data not shown). IL-10 secretion was significantly increased in transwell cocultures (Fig. 3*a*) and in separate experiments by H69 CM (Fig. 3*b*) in a dose-dependent fashion. However, the addition of anti-IL-10R-blocking antibody failed to reverse the inhibition in both transwell (Fig. 3*c*) and CM experiments (Fig. 3*d*).

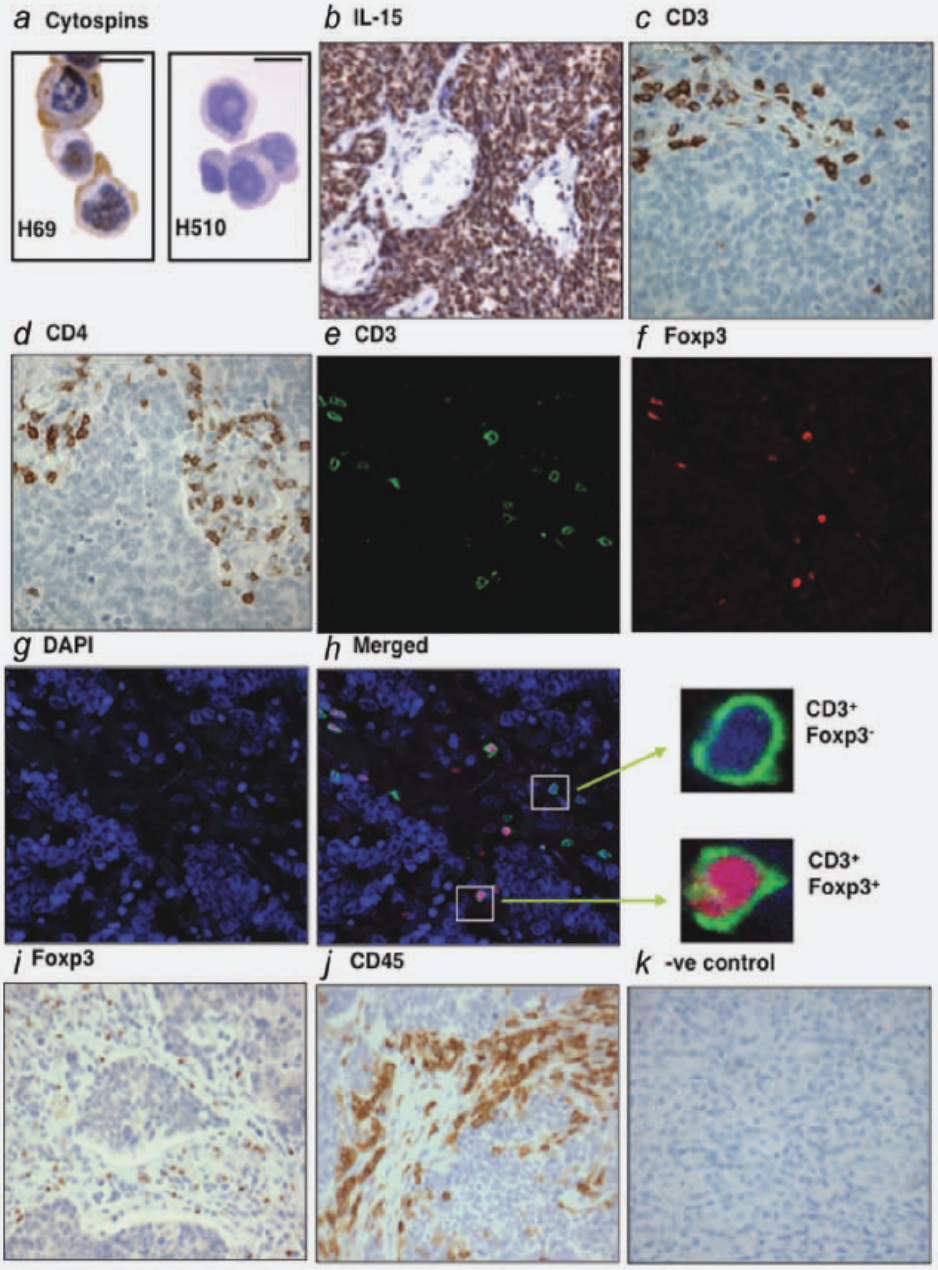

Figure 5. SCLC tumour cells express IL-15 and are infiltrated by Treg cells. (*a*) Immunohistochemistry showing IL-15 inside H69 but not H510 SCLC tumour cell lines. (*b–d*) Immunohistochemistry of representative SCLC biopsy sections showing (*b*) IL-15 inside tumour cells, (*c*) $CD3^+$ T cells and (*d*) $CD4^+$ T cells. (*e–h*) Immunofluorescence showing SCLC section simultaneously stained for (*e*) $CD3^+$ T cells, (*f*) FOXP3 transcription factor, (*g*) all nucleated cells (DAPI) and (H) merged stains and high power of merged stains showing the presence of $CD3^+$ T cells double stained for FOXP3. (*i–k*) Immunohistochemistry stained for (*i*) FOXP3 transcription factor, (*j*) CD45 leukocytes and (*k*) negative control.

IL-15 is produced by H69 SCLC cells and contributes to the induction of functional iTreg cells

To determine which components of CM inhibited $CD4^+$ T-cell proliferation, differential column filtration was carried out. The molecules involved were <30 kDa (Fig. 4*a*), which suggested that soluble cytokines might be candidates. As H510 SCLC tumour cells did not suppress T-lymphocyte proliferation (Fig. 1*a*), we compared publicly available micro-array data on cytokine gene expression in H69 cells, which inhibited proliferation relative to H510 cells which did not. This identified a number of cytokine genes (*IL-1a*, *IL-11*, *IL-15*, *IL-16*, *BMP-7*, *CSF-2* and *TGF-β2*) upregulated in H69 cells. As we had ruled out TGF-β signalling, of the remainder, *IL-15* was of particular interest for further investigation, as this has previously been shown to be involved in Treg cells induction.[26,27] We found that H69 cell lysates contained

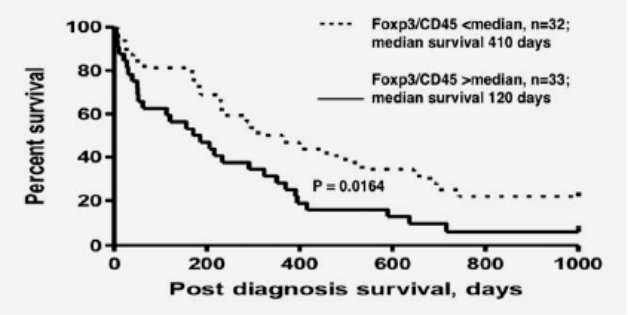

Figure 6. Poor patient survival correlates with a high intratumoural Treg cells:leukocyte ratio. Survival curve showing that for 65 patients diagnosed with SCLC, poor survival time correlated with having a high number of Treg cells in the intratumoural leukocyte infiltrate.

much higher levels of IL-15 protein than those from H510 cells (Fig. 4*b*). Anti-IL-15 immunostaining was present in H69 but not in H510 SCLC cells (Fig. 5*a*). Blocking IL-15 activity partially reversed H69 CM-induced inhibition of proliferation (Fig. 4*c*) and reduced $CD4^+$ T-cell secretion of IL-10 (Fig. 4*d*). Importantly, IL-15 increased FOXP3 population from naïve $CD4^+$ T cells activated by CD3/CD28 ligation in the context of H69 cells (Fig. 4*e*).

Tumour cells from patients with SCLC contain IL-15, and biopsies are infiltrated by Treg cells

In view of these results, we investigated IL-15 and Treg cell presence in biopsies from patients with SCLC. Critically, malignant cells in tumour biopsies contain IL-15 protein (Fig. 5*b*). Although considerably fewer in number than tumour cells, $CD3^+$ and $CD4^+$ T cells were present within the stroma of SCLC tumours (Figs. 5*c* and 5*d*). Immunofluorescence (Figs. 5*e*–5*h*) demonstrated that some $CD3^+$ T cells, but none of the tumour cells, were also positive for nuclear FOXP3.

Increased proportion of Treg cells in SCLC tumour biopsy infiltrates negatively correlates with patient survival

We examined the relationship between Treg cells in leukocyte infiltrates of SCLC tumour biopsies and patient survival retrospectively in 65 'typical' cases of SCLC. As we had shown that no tumour cells expressed FOXP3, we stained parallel sections from the biopsies with anti-FOXP3 (nuclear staining; Fig. 5*i*) and anti-CD45 (pan-leukocyte marker; Fig. 5*j*). Sections were counted blindly under 400× magnification to determine the ratio of $FOXP3^+$:$CD45^+$ cells as a measure of proportion of Treg cells in the infiltrating leukocyte population. Ten random high-power fields were counted per stain per biopsy, and the score per field was averaged. The number of $FOXP3^+$ cells counted per field ranged from 1 to 40, median 8 per high-power field; the number of $CD45^+$ leukocytes counted per field ranged from 3 to 127, median 37 per high-power field. The ratio of $FOXP3^+$:$CD45^+$ per field ranged from 1:33 to 1:1.25, median 1:3.7.

The effect on patient survival time was determined using Kaplan–Meier analysis. Critically, patients with $FOXP3^+$:$CD45^+$ ratios above the median value had significantly worse survival at 1,000 days post-diagnosis with a median survival time of 120 days compared to a median survival time of 410 days in the group with fewer $FOXP3^+$ cells in their leukocyte infiltrates (Fig. 6).

Discussion

Immunotherapy for solid tumours has focussed on amplification of intratumoural natural killer (NK) cells, natural killer T (NKT) cells and $CD8^+$ T-lymphocyte effector cell responses with limited success.[28–31] Evidence suggests that $CD4^+$ T cells, which are essential for generation of effective acquired immune responses to pathogens, are also important for anti-tumour immunity.[11]

We assessed whether SCLC tumour cells could modulate responses of $CD4^+$ T cells from healthy donors. We show that some SCLC tumour cell lines (H69 and H345 but not H510) inhibit proliferation of activated $CD4^+$ T cells. The inhibition induced by H69 cells was dose dependent, did not require cell–cell contact and was accompanied by functional $CD4^+CD25^+FOXP3^+CD127^{lo}Helios^-$ *de novo* Treg cell differentiation and IL-10 secretion. The inhibiting activity secreted by H69 cells had a molecular weight <30 kDa, which suggested that cytokines might be candidate molecules. Through interrogation of publicly available microarray data, we found that several cytokine genes were upregulated in H69 cells. These included *IL-15*, which is known to be involved in Treg cell induction.[26,27,32] The H69-induced inhibition of healthy $CD4^+$ T-cell proliferation was independent of IL10R and TGF-β-mediated signalling but was partially due to IL-15. IL-15 protein was present in H69, but not in H510 cells, and was found in tumour cells of SCLC biopsies.

IL-15 (MW = 14 kDa) belongs to the common gamma-chain (γc) cytokine family, is secreted by a variety of cell types,[33,34] binds to CD25[33] and modulates the biology of NK cells, NKT cells, $CD8^+$ T-cells, memory T-cells, monocytes and macrophages.[35,36] For these reasons, IL-15 has been promoted as a potential anti-tumour therapy to boost immune responses against cancer cells.[37] Our data suggest that IL-15 may have other effects in SCLC where it may potentiate the observed immune suppression associated with the disease by promoting Treg cells induction.

We investigated the relevance of our results to survival in SCLC. An excess of Treg cells may lead to a failure of tumour immunosurveillance and contribute to progression. $FOXP3^+$ Treg cells have been identified in a number of tumours.[19–25,38] However, the effect of $FOXP3^+$ T-lymphocyte infiltration on patient survival is controversial and appears to be tumour dependent. $FOXP3^+$ cell infiltration has been reported to have no prognostic effect in cutaneous malignant melanoma[20] or prostate cancer,[21] to be associated with improved survival in urinary bladder[22] and colon cancers[23,39] and with poor survival in uveal melanoma[24] and

gastric cancer.[25] These discordant effects suggest that tumour types must be investigated critically rather than ascribing a generalised function to Treg cells in cancer. We analysed 65 archival biopsies from patients with typical SCLC for which clinical details were available. To eliminate that the number of FOXP3$^+$ cells present in a tumour was simply a reflection of overall leukocyte infiltration, the ratio of FOXP3$^+$ cells in the leukocyte infiltrate was determined by staining parallel sections of needle lung biopsies from SCLC tumours for CD45, which marks all leukocytes, and for FOXP3. The results showed that a ratio of FOXP3$^+$ cells in the leukocyte infiltrate above the median value predicted poor survival. There was no difference in age, gender, disease stage or treatments between the groups (data not shown), suggesting that the ratio of FOXP3$^+$ cells in the leukocyte infiltrate can be regarded as an independent prognostic indicator in SCLC. Taken together, these results suggest that Treg cells and IL-15 are potential new therapeutic targets that may improve survival in this aggressive lung cancer.

Acknowledgements

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References

1. Rintoul RC, Sethi T. The role of extracellular matrix in small-cell lung cancer. *Lancet Oncol* 2001;2:437–42.
2. Oze I, Hotta K, Kiura K, et al. Twenty-seven years of phase III trials for patients with extensive disease small-cell lung cancer: disappointing results. *PLoS One* 2009;4:e7835.
3. Darnell RB, DeAngelis LM. Regression of small-cell lung carcinoma in patients with paraneoplastic neuronal antibodies. *Lancet* 1993; 341:21–2.
4. Nakamura H, Saji H, Ogata A, et al. Immunologic parameters as significant prognostic factors in lung cancer. *Lung Cancer* 2002;37: 161–9.
5. Johnston-Early A, Cohen MH, Fossieck BE, Jr et al. Delayed hypersensitivity skin testing as a prognostic indicator in patients with small cell lung cancer. *Cancer* 1983;52:1395–400.
6. Moriya K, Wakabayashi A, Shimizu M, et al. Induction of tumor-specific acquired immunity against already established tumors by selective stimulation of innate DEC-205(+) dendritic cells. *Cancer Immunol Immunother* 2010;59:1083–95.
7. Schwartz-Albiez R, Monteiro RC, Rodriguez M, et al. Natural antibodies, intravenous immunoglobulin and their role in autoimmunity, cancer and inflammation. *Clin Exp Immunol* 2009;158 (Suppl 1):43–50.
8. Nanni P, Nicoletti G, Palladini A, et al. Antimetastatic activity of a preventive cancer vaccine. *Cancer Res* 2007;67:11037–44.
9. Goldstein DJ, Austin JH, Zuech N, et al. Carcinoma of the lung after heart transplantation. *Transplantation* 1996;62:772–5.
10. Stebbing J, Duru O, Bower M. Non-AIDS-defining cancers. *Curr Opin Infect Dis* 2009;22: 7–10.
11. Hung K, Hayashi R, Lafond-Walker A, et al. The central role of CD4(+) T cells in the antitumor immune response. *J Exp Med* 1998;188:2357–68.
12. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 2009;30:646–55.
13. Banham AH, Powrie FM, Suri-Payer E. FOXP3+ regulatory T cells: current controversies and future perspectives. *Eur J Immunol* 2006;36: 2832–6.
14. Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 2006; 203:1693–700.
15. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 2006;203:1701–11.
16. Thornton AM, Korty PE, Tran DQ, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 2010;184:3433–41.
17. Gottschalk RA, Corse E, Allison JP. Expression of helios in peripherally induced foxp3+ regulatory T cells. *J Immunol* 2012;188:976–80.
18. Akimova T, Beier UH, Wang L, et al. Helios expression is a marker of T cell activation and proliferation. *PLoS One* 2011;6:e24226.
19. Woo EY, Chu CS, Goletz TJ, et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 2001;61: 4766–72.
20. Ladanyi A, Mohos A, Somlai B, et al. FOXP3+ cell density in primary tumor has no prognostic impact in patients with cutaneous malignant melanoma. *Pathol Oncol Res* 2010;16:303–9.
21. Sorrentino C, Musiani P, Pompa P, et al. Androgen deprivation boosts prostatic infiltration of cytotoxic and regulatory T lymphocytes and has no effect on disease-free survival in prostate cancer patients. *Clin Cancer Res* 2011;17:1571–81.
22. Winerdal ME, Marits P, Winerdal M, et al. FOXP3 and survival in urinary bladder cancer. *BJU Int* 2011;108:1672–8
23. Lee WS, Park S, Lee WY, et al. Clinical impact of tumor-infiltrating lymphocytes for survival in stage II colon cancer. *Cancer* 2010;116:5188–99.
24. Mougiakakos D, Johansson CC, Trocme E, et al. Intratumoral forkhead box P3-positive regulatory T cells predict poor survival in cyclooxygenase-2-positive uveal melanoma. *Cancer* 2010;116: 2224–33.
25. Shen Z, Zhou S, Wang Y, et al. Higher intratumoral infiltrated Foxp3+ Treg numbers and Foxp3+/CD8+ ratio are associated with adverse prognosis in resectable gastric cancer. *J Cancer Res Clin Oncol* 2010;136:1585–95.
26. Xia J, Liu W, Hu B, et al. IL-15 promotes regulatory T cell function and protects against diabetes development in NK-depleted NOD mice. *Clin Immunol* 2010;134:130–9.
27. Imamichi H, Sereti I, Lane HC. IL-15 acts as a potent inducer of CD4(+)CD25(hi) cells expressing FOXP3. *Eur J Immunol* 2008;38: 1621–30.
28. Motohashi S, Nakayama T. Clinical applications of natural killer T cell-based immunotherapy for cancer. *Cancer Sci* 2008;99:638–45.
29. Geller MA, Cooley S, Judson PL, et al. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. *Cytotherapy* 2011;13: 98–107.
30. Dudley ME, Gross CA, Langhan MM, et al. CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clin Cancer Res* 2010;16: 6122–31.
31. Rowley J, Monie A, Hung CF, et al. Inhibition of tumor growth by NK1.1+ cells and CD8+ T cells activated by IL-15 through receptor β/common γ signaling in trans. *J Immunol* 2008; 181:8237–47.
32. Casetti R, Agrati C, Wallace M, et al. Cutting edge: TGF-β1 and IL-15 induce FOXP3+ γδ regulatory T cells in the presence of antigen stimulation. *J Immunol* 2009;183:3574–7.
33. Grabstein KH, Eisenman J, Shanebeck K, et al. Cloning of a T cell growth factor that interacts with the β chain of the interleukin-2 receptor. *Science* 1994;264:965–8.
34. Podolsky DK. Healing the epithelium: solving the problem from two sides. *J Gastroenterol* 1997;32: 122–6.
35. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* 2006;6: 595–601.
36. Badolato R, Ponzi AN, Millesimo M, et al. Interleukin-15 (IL-15) induces IL-8 and monocyte chemotactic protein 1 production in human monocytes. *Blood* 1997;90:2804–9.
37. Jakobisiak M, Golab J, Lasek W. Interleukin 15 as a promising candidate for tumor immunotherapy. *Cytokine Growth Factor Rev* 2011;22:99–108.
38. Lindqvist CA, Christiansson LH, Thorn I, et al. Both CD4+ FoxP3+ and CD4+ FoxP3− T cells from patients with B-cell malignancy express cytolytic markers and kill autologous leukaemic B cells in vitro. *Immunology* 2011; 133:296–306.
39. Correale P, Rotundo MS, Del Vecchio MT, et al. Regulatory (FoxP3+) T-cell tumor infiltration is a favorable prognostic factor in advanced colon cancer patients undergoing chemo or chemoimmunotherapy. *J Immunother* 2010;33: 435–41.